Coping with ephemeral resources and unpredictable hosts: nitrogen allocation in the post-fire annual *Nicotiana attenuata*, and a plant-specific pheromone used by its seed predator *Corimelaena extensa*

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To my first and most important teachers:

Virgilia and **Michael Stanton.**

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1. Introduction

1.1. Phenotypic plasticity in plant-insect interactions

Plants are the base of terrestrial ecosystems fixing solar energy through photosynthesis and are therefore constantly under the threat of losing biomass to primary consumers, which range from vertebrates to invertebrates with a large variety of feeding behaviours. Among these consumers, insects represent one of the oldest threats, first appearing in the fossil record approximately 400 mya and shortly thereafter starting to feed on plants (Labandeira, 1998; Engel and Grimaldi, 2004). Indeed, the extensive radiation and evolutionary success of many clades of the Class Insecta are thought to be associated to their phytophagous habits, and herbivorous insects are found in 8 of 32 insect orders and represent more than a quarter of all living organisms (Romoser and Stoffolano, 1998). The phytophagous feeding habits in these clades range from use of chewing mouthparts in both juveniles and adults (in the Orthoptera, Phasmida, Coleoptera and Hymenoptera); through clades that use chewing mouthparts only in the juvenile stages (Diptera and Lepidoptera); to groups that have piercing-sucking mouthparts in the Thysanoptera and Hemiptera. Piercing-sucking herbivorous habits comprise cell-content feeders, phloem- and xylem-feeders, and lacerate-and-flush feeders – the latter of which usually consume nutrient-rich mesophyll or parenchyma cells from storage or reproductive organs (Romoser and Stoffolano, 1998; Weirauch and Schuh, 2011).

Plants are not passive targets of herbivore damage, and their counter adaptations include physical defences such as trichomes, thorns and thickened cuticles; and also a variety of metabolites which act as anti-digestives, repellents or toxins to feeding animals, for e.g., phenolics, terpenes, proteinase inhibitors and alkaloids (Karban and Baldwin, 1997; Howe and Jander, 2008). Functionally, plant defences can also be classified into direct defences which act upon the herbivore itself and have a toxic or anti-digestive effect, and indirect defences, which attract natural enemies of feeding herbivores to the site of attack (reviewed in Karban and Baldwin, 1997). Defences may either be constitutive, those that are present in plant tissue before herbivore attack; or induced, those that are produced only after damage and elicitation by herbivore feeding. Although these classifications are helpful to understand the large diversity of plant defence mechanisms, in reality there is a gradient in defence types

and single defences may belong to more than one of these classes. For example, glandular trichomes combine the physical aspect of acting as a barrier to decrease herbivore access to leaf surfaces, with chemical compounds that can act as toxins and even as indirect defences (Van Dam and Hare, 1998; Simmons and Gurr, 2004; Weinhold and Baldwin, 2011). Similarly, nicotine is the main defensive alkaloid in the wild tobaccos *Nicotiana attenuata* and *N. sylvestris* and it is present constitutively throughout the plant, but is also inducible in both species after herbivore attack (Baldwin, 1999). Since insect orders which show phytophagous behaviour are the most diverse groups within class Insecta, many of the myriad plant anti-herbivore adaptations are thought to have evolved in response to plant-insect interactions (Fraenkel, 1959; Ehrlich and Raven, 1964; Stamp, 2003), and these interactions constitute the broad topic of the present dissertation.

The capacity of an individual to respond to diverse environmental cues in a plastic manner is considered essential for survival in variable habitats (Agrawal, 2001) and induced plant defences are a widespread example of adaptive phenotypic plasticity, allowing plants to adjust to changes in the identity, abundance and timing the herbivore community (Karban and Baldwin, 1997; Karban, 2011). In order to coordinate the induction of anti-herbivore defences, plants use the evolutionarily conserved jasmonic acid (JA) signalling cascade. This phytohormone has been shown to induce direct and indirect defences in a large variety of plant species (reviewed in Howe and Jander, 2008) and these responses are activated by elicitors present in the oral secretions (OS) of many insect species. Examples of elicitors in insect OS include fatty acid-amino acid conjugates (FACs) and inceptins in Lepidoptera, and caeliferins in Orthoptera (Alborn *et al.*, 1997; Halitschke *et al.*, 2001; Schmelz *et al.*, 2006; Alborn *et al.*, 2007). Additionally some plants have been shown to respond to elicitors present in the oviposition fluids of some insects (e.g. Hilker and Meiners, 2006) or oviposition damage (Puysseleyr *et al.*, 2010). This large plasticity in plant metabolism can affect the abundance, distribution and performance of herbivores and their natural enemies, ultimately influencing community structure (Agrawal, 2001; Karban, 2011).

1.2.*Nicotiana attenuata* as a model for studying plasticity in resource allocation to chemical defences

Many of the explanations for the widespread use of induced defences by plants hinge on their costs (for a review see Stamp, 2003). Trade-offs between defences against different types of herbivores or between defences against herbivores and pollinator attraction are examples of ecological costs of defences (Strauss *et al.*, 2002). Allocation costs are also expected to be important in the evolution of induced defences, since resources invested into defences cannot be simultaneously invested into growth and reproduction. Resource limitation is thought to further constrain this plasticity and increase the costs of defences (Coley *et al.*, 1985; Bazzaz *et al.*, 1987; Chapin III, 1991) and the expression of defences can be altered by limitations on resources. Some examples of resource limitations decreasing production of defences include the induction of nicotine in wild and cultivated tobacco, of proteinase inhibitors and phenolics in wild tobacco and cyanogenic glycosides in eucalyptus trees (Matt *et al.*, 2002; Lou and Baldwin, 2004; Simon *et al.*, 2010). Several studies have shown costs of inducing defences such as decreased seed set and competitive ability in *N. attenuata* caused by JA-induced responses (Baldwin, 1998; van Dam and Baldwin, 1998) and also decreased seed set in *A. thaliana* and reduced seed set and biomass gain in tomato after induction of JA-responses (Cipollini, 2002; Redman *et al.*, 2001). And molecular biology tools have enabled the analysis of costs of single defences or single classes of defences, such as reduced growth and seed set caused by trypsin proteinase inhibitors (TPIs) in *N. attenuata* (Zavala *et al.*, 2004) or reduced growth in *Arabidopsis thaliana* due to glucosinolate induction (Züst *et al.*, 2011). But the measurement of costs as reductions in biomass does not distinguish between the allocation of resources to single growth-related compounds, such as photosynthetic proteins, or to defence-related compounds (Chapin III *et al.*, 1990). Furthermore, trade-offs between growth and defence are likely to only be apparent when measuring the allocation of a growth limiting resource such as nitrogen (N) (Mole, 1994; Baldwin *et al.*, 1998; van Dam and Baldwin, 1998; Heil and Baldwin, 2002).

In the first part of this dissertation, I measured the plasticity in N allocation between growth and N-based defences in the wild tobacco tobacco *Nicotiana attenuata* Torr. Ex Watts (Solanaceae). *N. attenuata* is a post-fire annual native to the Great Basin Desert in the south western USA, which synchronizes its germination from long-lived seed banks in response to cues from pyrolysed vegetation (Preston and Baldwin 1999). Due to its mass germination

behaviour in transiently N-rich soils, *N. attenuata* faces intense intra-specific competition for this growth-limiting resource. Additionally, as a pioneer species in this habitat, it is attacked by a diverse and unpredictable community of herbivores from different feeding guilds (Fig. 1), among which are larvae of the specialist tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae, Fig 1B), whose interaction with *N. attenuata* has been extensively studied. Fatty acid-amino acid conjugates in the oral secretions of *M. sexta* larvae activate the jasmonic acid (JA) signalling cascade (Kessler *et al.*, 2004; Halitschke *et al.*, 2001) and downstream responses; and the application of diluted *M. sexta* OS to leaf wounds are sufficient to elicit similar responses as herbivore attack. JA signalling activates JA-responsive transcription factors that lead to the biosynthesis of a plethora of induced small metabolites (reviewed in Woldemariam *et al.*, 2011), such as the N-intensive alkaloid, nicotine, and a variety of phenolamides, which decrease herbivore performance (Baldwin 1999, Kaur *et al.* 2010, Onkokesung *et al.* 2010, Steppuhn *et al.* 2004). Nicotine is present constitutively in undamaged *N. attenuata* tissues, but increases inducibly in response to damage (Baldwin *et al.* 1998, Baldwin 1999). The two major phenolamides found in *N. attenuata* are the N-acylated polyamines caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS), for which biosynthesis is regulated by the transcription factor, NaMYB8 (MYB8, Figure 2). Both CP and DCS accumulate constitutively in reproductive tissues and are strongly induced by herbivory in leaves (Kaur *et al.* 2010). Herbivory also causes large-scale changes in *N. attenuata*'s transcriptome and proteome, decreasing levels of photosynthetic genes and proteins, including RuBisCO (Giri *et al.* 2006, Halitschke *et al.* 2003, Voelckel and Baldwin 2004). Due to the importance of N for its growth and defence in the post-fire environment, as well as the large plasticity of its responses to herbivory, and the availability of previously described isogenic transgenic lines impaired in individual classes of defences, *N. attenuata* is an ideal model in which to study growth-defense trade-offs in a common N currency.

To analyse the growth-defence trade-offs between single compounds, a stable isotope (^{15}N) pulse was given to soil-grown plants modified from Van Dam and Baldwin (2001). High throughput liquid chromatograph-mass spectrometry (LC-MS) methods have been developed which can be used for the analysis of ^{15}N incorporation into small metabolites (e.g. Gaquerel *et al.*, 2010), but although (MS)-based methods have been used for measurements of *in vivo* ^{15}N incorporation into proteins (MacCoss *et al.*, 2005; Snijders *et al.*, 2005; Taubert *et al.*, 2011; Jehmlich *et al.*, 2008), separation of complex protein samples still

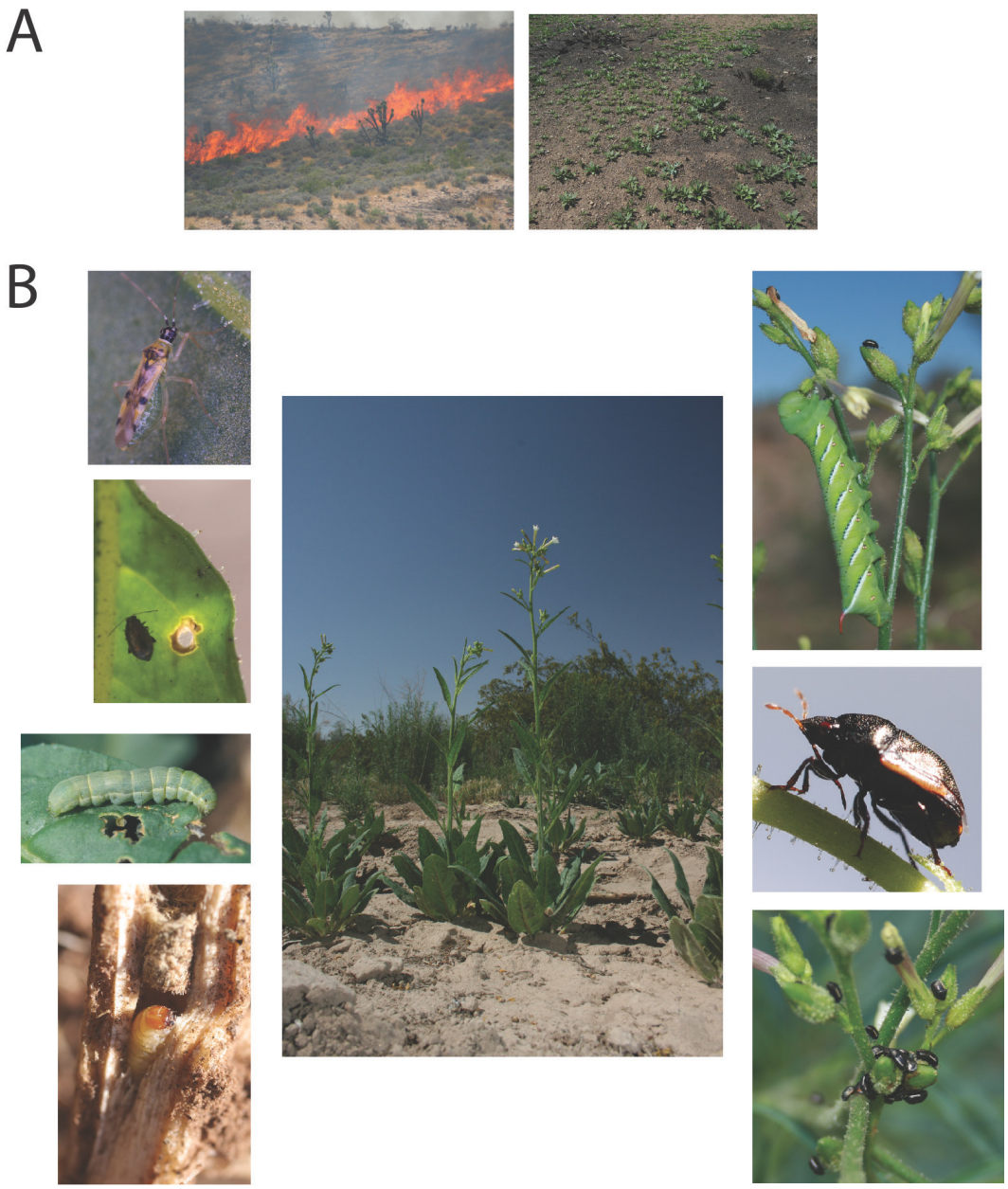

Figure 1. A) The wild tobacco *N. attenuata* germinates synchronously after wild-fires from long-lived seed banks (photos D. Kessler). **B)** As a pioneer in the post-fire environment *N. attenuata* (centre) can be attacked by a large variety of herbivores. On the left, from the top, the mirid *Tupiocoris notatus* (A. Kessler), the flea beetle *Epitrix subcrinita* (A. Steppuhn), larvae of the noctuid *Spodoptera exigua* (D. Kessler), larvae of the stem borer *Trichobaris muccorea* (A. Steppuhn). On the right side, from the top, larvae of the sphingid *Manduca sexta* (D. Kessler), the thyreocorid bug *Corimelaena extensa* (A. Van Doorn) and an aggregation of *C. extensa* on flowers and seed capsules of *N. attenuata* (D. Kessler).

heavily rely on 2D-gell electrophoresis. In **manuscript I**, I collaborated on the development of a high throughput LC-MS^E method for separation, quantification and ^{15}N incorporation measurement of proteins extracted from pulse-labelled soil-grown plants, which enables an ecologically realistic measurement of growth-defence trade-offs. This method allowed for a direct comparison of N investment into proteins with a similar accuracy as the LC-UV-MS method used for quantification and ^{15}N incorporation measurement of defensive metabolites, despite the large mass difference between these two classes of molecules. I then applied this new method to study the influence of the JA signalling cascade and the biosynthesis of phenolamides on growth-defence trade-offs after simulated *M. sexta* herbivory in *N. attenuata*, in **manuscript II**. In this chapter and the next, I use the photosynthetic protein ribulose-1,5-bisphosphate-carboxylase/ oxygenase (RuBisCO) as a proxy for N investment into plant growth and compare this to the investment into the three major N-based defence metabolites of this species, nicotine, CP and DCS. The photosynthetic protein ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO) was used as a proxy for N investment into growth, since it is the most abundant foliar protein in plants and a putative N storage protein (Ellis, 1979; Millard, 1988). Additionally, RuBisCO is directly involved in the dark cycle of photosynthesis and changes in RuBisCO regulation are associated with changes in growth and N metabolism (Millard and Catt, 1988; Stitt and Schulze, 1994; Smith and Stitt, 2007).

In **manuscript II**, I use a previously described transgenic line silenced for the first step of JA biosynthesis by an inverted repeat (ir) RNAi construct, hereafter ir*LOX3* plants (Allmann *et al.*, 2010) to further explore the effects of JA signalling on N allocation after simulated herbivory. I also evaluate the effect of phenolamide biosynthesis by using another transgenic line silenced for the expression of the transcription factor, Na*MYB8*, which controls phenolamide biosynthesis, hereafter ir*MYB8* plants (Kaur *et al.*, 2010). Finally, in **manuscript III**, I use a transgenic line silenced in the expression of RuBisCO via an antisense construct, hereafter as*RUB* plants (Mitra and Baldwin, 2008), to support the conclusions of the previous chapter that N for CP and DCS biosynthesis is not derived from RuBisCO degradation, but likely from recently assimilated N. A simplified overview of the JA-induced plasticity in N allocation of *N. attenuata* and the experimental setup used in this part of the dissertation can be seen in Fig. 2.

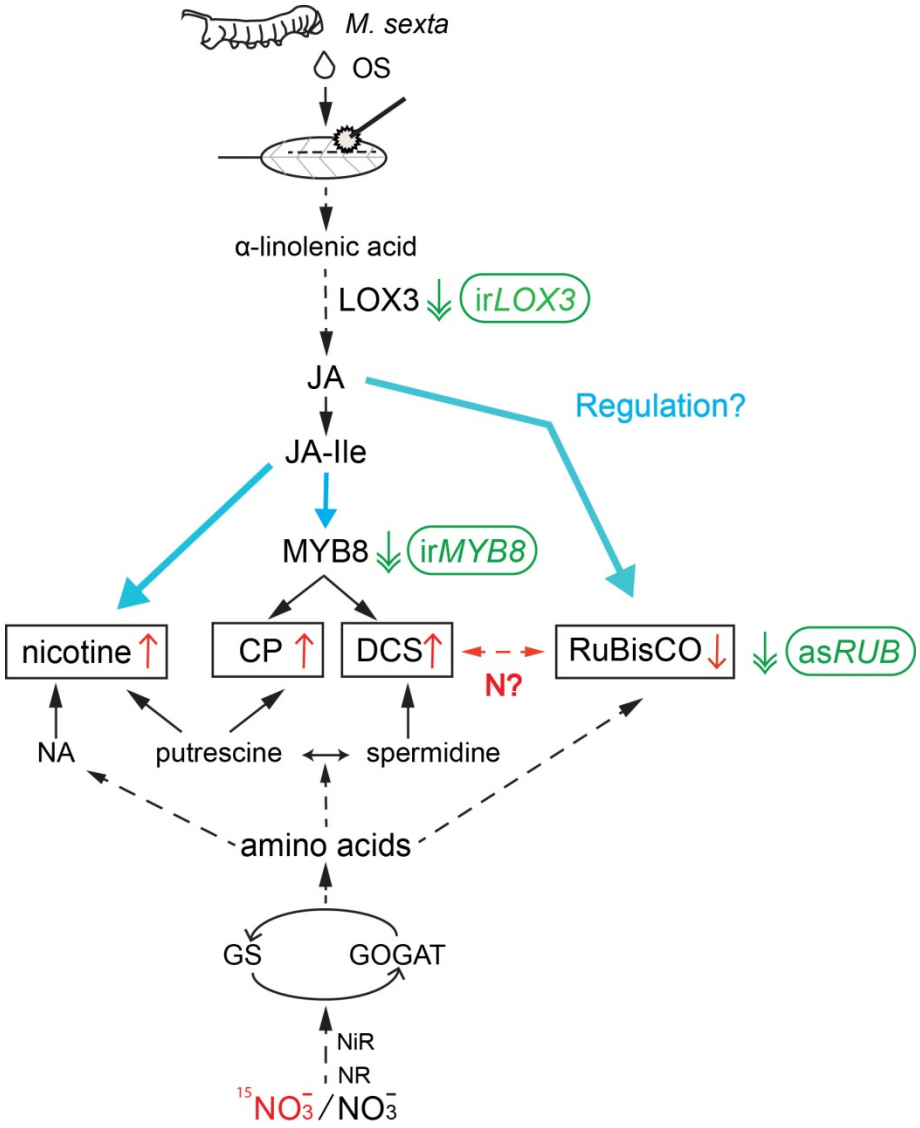

Figure 2. Overview of experimental strategy used to study growth-defense trade-offs in *Nicotiana attenuata* in a common nitrogen (N) currency in manuscripts II and III. The biosynthesis of nicotine, caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS) is induced after simulated herbivory in wild type (WT) by wounding (W) with a pattern wheel and application of oral secretions (OS) of *Manduca sexta*, but is impaired in the transgenic plants silenced in the expression of lipoxygenase 3 (LOX3) or MYB8 by RNAi with inverted-repeat (ir) constructs in **manuscript II**. The concentration of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) decreases in WT after W+OS, but the effects of jasmonic acid (JA) on N-investment into RuBisCO are unclear and were studied in **manuscript III** with a line silenced for RuBisCO expression with an antisense construct (as). Amino acids serve as precursors for putrescine and spermidine and for nicotinic acid (NA), which provide N for the synthesis of these metabolites. Amino acids are derived from nitrate (NO_3^-) reduction, followed by assimilation catalyzed by glutamine synthetase (GS) and glutamate synthase (GOGAT), and are also used as precursors for RuBisCO synthesis. JA-Ile=JA- isoleucine; NR=nitrate reductase; NiR=nitrite reductase. Red arrows represent measured N incorporation points, blue arrows represent regulatory pathways, black arrows represent biosynthetic pathways and green arrows represent transgenic silencing of a gene.

1.3. Plant-mediated plasticity in the host colonization of *Nicotiana* spp. by the seed predator *Corimelaena extensa*

Apart from affecting their performance, plasticity in plant metabolism can also affect herbivore behaviour at multiple scales (Karban, 2011). Within plant differences in metabolite induction and distribution have been shown to cause feeding herbivores to move away from locally induced leaves to avoid defences (Rodriguez-Saona and Thaler, 2005; Underwood *et al.*, 2005) or cause attraction of herbivores to feeding sites, as in the larvae of *Spodoptera frugiperda* (Carroll *et al.*, 2008). At the whole plant scale, induced changes in plant volatiles can act as infochemicals, affecting host plant location by changing plant apparency to herbivores (Dicke, 2000; Dicke and Baldwin, 2010). These infochemicals have been shown to increase attraction of herbivores to some host plants (Blackmer *et al.*, 2004; Halitschke *et al.*, 2008), but can also decrease oviposition in other species, as has been shown for *Manduca sexta* moths on *Datura wrightii* (Allmann *et al.*, 2013) and in many Pentatomorphan true bugs (Martinez *et al.*, 2013). Finally, at a coarser scale, herbivore attraction or repellence to a plant can also be affected by its neighbouring plants in a patch, which may effectively hide or expose it based at least in part on plant chemical cues (Barbosa *et al.*, 2009) increasing habitat heterogeneity.

Many of the studies of the effects of variation in host plant chemistry affecting host choices, have focused on oviposition choices by adult Lepidoptera, which are thought to seek hosts that maximize the performance of their offspring according to the "preference-performance hypothesis" (Jaenike, 1978; Landolt and Phillips, 1997; Scheirs and Bruyn, 2002). Since lepidopteran larval stages are not very mobile, especially in their early life, and usually spend most of their development on the same individual host plant, a female's host choice is especially important in determining larval survival and development (Martinez *et al.*, 2013). For phytophagous insects that feed both as adults and juveniles on the same host plant and which must colonize their host plant after overwintering elsewhere, plasticity in plant chemistry may influence host finding and preference, but also adult performance (Scheirs and Bruyn, 2002; Martinez *et al.*, 2013). Furthermore, in the case of a non-apparent host such as the fire-chasing *N. attenuata*, the host plant itself represents a resource which is scattered in space and time, and this could also interact with plasticity in plant chemistry to increase habitat heterogeneity for foraging insects.

One behavioural adaptation of herbivorous insects to reduce the variability in plant-derived signals and increase host apparency is the use of insect-derived infochemicals, such as pheromones. While sexual pheromones have a primary reproductive function and are characterized by single emitters attracting mates (Schlyter and Birgersson, 1989; Landolt and Phillips, 1997), the use of aggregation pheromones is usually characterized by collective signalling which leads to formation of groups which may have multiple functions apart from reproduction (Shorey, 1973; Wertheim *et al.*, 2005). Although many pheromones have been characterized in various insect taxa, the study of these infochemicals in a cost-benefit framework is still far less developed than the advances in chemical elucidation of these compounds and development of applications for biological control (Millar, 2005; Wertheim *et al.*, 2005). Unlike the variety of biosynthetic mechanisms and their chemical variety, the evolutionary ecology of these compounds is thought to be common among different insect taxa (Tillman *et al.*, 1999; Wertheim *et al.*, 2005) and is discussed in further detail in **manuscript IV** where I characterize the interaction of *N. attenuata* with a hemipteran seed feeder, *Corimelaena extensa*.

Benefits of the use of pheromones to form conspecific aggregations may include overcoming of host plant defences, increased protection from predators, more efficient resource use, better mate finding and improved localized abiotic conditions (reviewed in Wertheim *et al.*, 2005). Many of these beneficial traits have been shown in the interactions of many species of bark beetles (Coleoptera: Scolytinae) and their conifer hosts, in which beetles use a concerted attack mediated by aggregation pheromones that overcomes the host's defenses allowing them to inoculate the trees with their symbiotic fungi, which provide nutrition for their larvae (reviewed in Wood, 1982; Byers, 1989), and recent studies have shown additional layers of complexity in the interaction between host plant chemicals with beetles and symbiotic fungi and bacteria which can conditionally increase or decrease the costs and benefits of each member (reviewed in Raffa, 2013). Furthermore, many of these pheromones are derived from host plant metabolites, indicating that plasticity in host plant metabolites can directly influence pheromone signal, although this has been investigated in few studies (but see Bashir *et al.*, 2003; Edde and Phillips, 2007; Aukema *et al.*, 2010). Examples of plant-derived pheromones include sexual pheromones derived from pyrrolizidine alkaloids in Arctiid moths (Eisner and Meinwald, 1995), terpenoid compounds in many bark beetle pheromone blends (Aukema *et al.*, 2010) and possibly also the

isothyocianate-derived components of the aggregation pheromone in *Phyllotreta striolata* (Beran *et al.*, 2011).

In **manuscript IV**, I characterize the host-finding behaviour of the seed feeding true bug *Corimelaena extensa* Uhler (Hemiptera: Thyreocoridae, Fig. 1B) which feeds on *N. attenuata* and the closely related perennial *N. obtusifolia* in the Great Basin Desert in Utah. Although *C. extensa* has been reported to feed on another wild tobacco species in California, *N. glauca* (Lung and Goeden, 1982), and attack by *Corimelaena* spp. has been shown to decrease seed mass and viability of *N. attenuata* (Baldwin and Morse, 1994; Baldwin *et al.*, 1997; Karban, 1997), little else is known of the ecology of these so called "negro" or "ebony" bugs and the interactions with their tobacco hosts. *C. extensa* adults emerging from overwintering form larger aggregations on *N. attenuata* than on *N. obtusifolia*, even though the former is a presumably less apparent host, due to its fire-chasing habit and to being an annual plant with a shorter flowering phenology. Fig. 3 shows a model of some of the host plant traits which may influence colonization of both hosts by *C. extensa* and its predictions. In this part of the dissertation I show, through identification of the aggregation pheromone of *C. extensa* and host transplantation experiments, that this insect exhibits a host plant-mediated plasticity in pheromone release and relies more heavily on the aggregation pheromone to find the unpredictable host plant species, *N. attenuata*, on which it realizes a greater fitness than when feeding on *N. obtusifolia.* The mechanisms through which these two host plants determine insect fitness still need to be dissected in further studies, although differences in plant chemistry are likely to be important.

In the **Appendix** I also show results from a *C. extensa* performance experiment with transgenic lines of *N. attenuata* independently silenced for release of floral scent, green leaf volatile emission, different steps of the JA signalling cascade and the two important direct defences, nicotine and trypsin proteinase inhibitors (TPI). None of these traits showed an effect on adult fecundity compared to WT, and a comparison *C. extensa* fed on two different accessions suggests that plant reproductive effort (number of buds, flowers and seed capsules) may be a better predictor of insect fecundity than JA-elicited defences. These results are supported by a field elicitation experiment with methyl-jasmonate, which activates JA-responses in *N. attenuata*. These additional data are further discussed in the general discussion at the end of this dissertation.

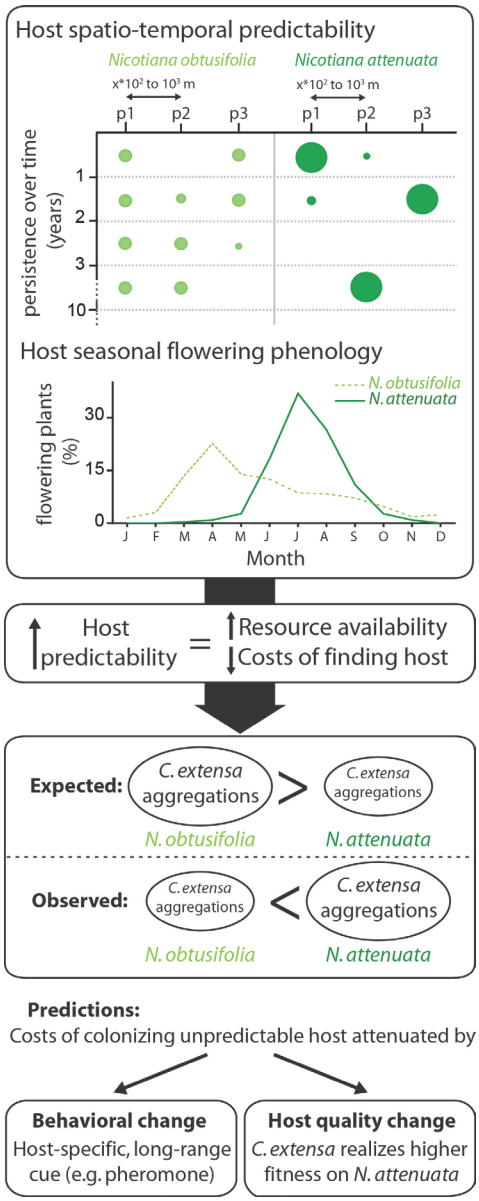

Figure 3. Overview of the study system and conceptual framework for host plant colonization by *Corimelaena extensa* in **manuscript IV**. Distances between patches (p1, p2, etc) of the host plants *Nicotiana obtusifolia* and *N. attenuata* is similar, but irregular post-burn *N. attenuata* patches tend to be larger than *N. obtusifolia* patches. The hosts also differ in their persistence over the years and flowering phenology. A host with higher predictability is assumed to provide more resources and to diminish dispersal costs (e.g., energy and time investment) of host finding. Therefore we expect *C. extensa* to form larger aggregations on *N. obtusifolia*, but the opposite is observed in the field. We predict that the colonization of *N. attenuata* by *C. extensa* is mediated by use of specific and reliable cues, such as a pheromone, and through changes in host quality which lead to higher fitness on this host, mitigating the costs of host finding.

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2. Manuscript Overview

Note: The formatting and terminology of published manuscripts were maintained and there may be minor differences in terminology between chapters.

Manuscript I

Determination of ^{15}N-Incorporation into Plant Proteins and their Absolute Quantitation: A New Tool to Study Nitrogen Flux Dynamics and Protein Pool Sizes Elicited by Plant−Herbivore Interactions

Lynn Ullmann-Zeunert, Alexander Muck*, Natalie Wielsch*, Franziska Hufsky, Mariana A. Stanton, Stefan Bartram, Sebastian Böcker, Karin Groten and Aleš Svatoš

*These authors contributed equally to the manuscript.

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In this manuscript we develop a high throughput LC-MS^E method which allows for simultaneous absolute quantification and calculation of ^{15}N incorporation into plant proteins. It also enables the measurement of soil-grown plants with an isotope-pulse labelling technique, instead of using stable labelling techniques which require a set of labelled and unlabelled samples. Although my contribution to this manuscript was small, I contributed to designing and performing the experiments and performed the LC-MS measurement of ^{15}N incorporation into defensive compounds to test for comparable accuracy between the incorporation rates measured in proteins using this novel method with that of the small N-containing defensive metabolites (e.g. nicotine). And since this method was crucial for the sample measurements and consequent ecological insights discussed in manuscripts II and III, this manuscript was included in my thesis.

Lynn Ulmann-Zeunert designed and performed the experiments, prepared IRMS measurements, did protein analysis and drafted the manuscript. Alexander Muck established the LC-MS^E measurements and processed half of the MS measurements. Natalie Wielsch revised the manuscript and processed the second half of the MS measurements. Franziska Hufsky programmed MoLE, and processed the extracted MS-spectra for further analysis. Mariana A. Stanton designed and performed experiments. Stefan Bartram processed IRMS measurements and gave helpful comments. Sebastian Böcker gave helpful comments for additional experiments and developed the final formula for protein quantitation. Ian T. Baldwin revised the manuscript and helped with design of the experiments. Karin Groten designed experiments, supported method development, and helped with the manuscript preparation Aleš Svatoš helped with method development, revised and submitted the manuscript.

Manuscript II

Quantification of growth-defense trade-offs in a common currency: nitrogen required for phenolamide defenses is not derived from ribulose-1,5-bisphosphate carboxylase/oxygenase turnover

Lynn Ullmann-Zeunert*, Mariana A. Stanton*, Natalie Wielsch, Stefan Bartram, Christian Hummert, Aleš Svatoš, Ian T. Baldwin and Karin Groten

* These authors contributed equally to the manuscript

Published in *The Plant Journal* 2013, **75**(3):417-429, doi: 10.1111/tpj.12210

In this manuscript, by using the novel LC-MS^E method described in manuscript I, we measured nitrogen (N) investment into growth a related protein and defensive N-containing metabolites in *Nicotiana attenuata*. Using a transgenic line silenced for jasmonate signalling and another silenced for the production of phernolamides we found that repeated simulated herbivory reconfigures N allocation at multiple scales in *N. attenuata* and that phenolamides regulate the reallocation of N from proteins. Decrease in total N of shoots suggested allocation to roots in WT plants and leaves of different developmental stage showed differences in their relative N allocation to defences and proteins. A ^{15}N-pulse labelling

experiment showed that N for the biosynthesis of phenolamides does not come from ribulose-1,5-bisphosphate carboxylase/ oxygenase, despite it being the most abundant foliar protein and having showed a sharp decrease after simulated herbivory.

Lynn Ulmann-Zeunert designed and performed the experiments, performed protein measurements, analysed the data and wrote the manuscript. Mariana A. Stanton designed and performed the experiments, performed metabolite analysis, analysed the data and wrote the manuscript. Natalie Wielsch and Aleš Svatoš processed the LC-MS^E analysis. Stefan Bartram processed the IR-MS analysis. Christian Hummert contributed with clustering statistical analysis and heatmaps. Ian T. Baldwin and Karin Groten designed the experiments, coordinated the research and revised the manuscript.

Manuscript III

Silencing ribulose-1,5-bisphosphate carboxylase/oxygenase expression does not disrupt nitrogen allocation to defense after simulated herbivory in *Nicotiana attenuata*

Mariana A. Stanton, Lynn Ullmann-Zeunert, Natalie Wielsch, Stefan Bartram, Aleš Svatoš, Ian T. Baldwin and Karin Groten

Published in *Plant Signaling and Behavior* 2013, **8**(12): e27570, doi: 10.4161/psb.27570

In this study, using the same experimental design as in manuscript II, we characterized the nitrogen (N) investment into N-containing defences and ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO) in a transgenic line of *Nicotiana attenuata* silenced for the photosynthetic protein RuBisCO (as*RUB*). Here we show that as*RUB* invests similar amounts of N into nicotine and caffeoyl-putrescine as WT and even larger amounts into dicaffeoyl-spermidine. This investment likely comes from the higher nitrate pools of as*RUB* supporting our previous observation that recently assimilated N is invested into rapidly induced defences after simulated herbivory and does not come from RuBisCO degradation. Additionally as*RUB* has similar soluble protein levels as WT which suggest N surplus from RuBisCO silencing is diverted to other proteins. We propose that the observed

decreased in leaf proteins observed in this study and in manuscript II are part of a plant tolerance mechanism after herbivory in which N from proteins may be rapidly reallocated to avoid ingestion by herbivores.

Mariana A. Stanton designed and performed the experiments, performed metabolite measurements, analysed the data and wrote the manuscript. Lynn Ulmann-Zeunert designed and performed the experiments, performed protein measurements and analysed the data. Natalie Wielsch and Aleš Svatoš assisted with LC-MS^E analysis. Stefan Bartram assisted with IR-MS analysis. Ian T. Baldwin designed the experiments and revised the manuscript. Karin Groten designed the experiments, coordinated the research and wrote the manuscript.

Manuscript IV

Plant-mediated plasticity in emission of an aggregation pheromone provides a reliable signal for a native tobacco seed feeder to locate an unreliable host plant in the field

Mariana A. Stanton, Jens Preβler, Christian Paetz, Wilhelm Boland, Aleš Svatoš and Ian T. Baldwin

Manuscript in preparation for submission

In this manuscript we characterize the host-finding behaviour of *Corimelaena extensa* true bugs which are seed predators of both the fire-chasing annual *Nicotiana attenuata* and the perennial *N. obtusifolia* in the Great Basin Desert in Utah (USA). We characterized and *de novo* synthesized the aggregation pheromone of *C. extensa* (5Z, 8Z)-tetradeca-5,8-dienal - and used the synthetic product to test its adaptive value in the colonization of both host plants. We show that *C. extensa* emits higher amounts of the pheromone and has higher fitness when feeding on *N. attenuata*, which may offset the higher dispersal costs of colonizing this less apparent host. We conclude that this newly discovered aggregation pheromone allows the insect to find the highly unpredictable host on which it realizes the greatest fitness, by changing the apparency of this host to conspecifics. We also discuss how host plant chemistry may affect this interaction. This is, to our knowledge, the first time a

pheromone is described for a species of the Thyreocoridae (Hemiptera) family and the first ecological study on this insect species.

Mariana A. Stanton designed and performed the lab and field experiments, analysed the data, isolated and contributed to the structural elucidation of the pheromone, and wrote the manuscript. Jens Preβler synthesized the pheromone and contributed to the structural elucidation. Christian Paetz performed NMR measurements and contributed to the structural elucidation. Wilhelm Boland coordinated the synthesis and contributed to the structural elucidation. Aleš Svatoš contributed to the MS-based structural elucidation. Ian T. Baldwin contributed to experimental design, performed field experiments, coordinated the research and revised the manuscript.

Manuscript I

Journal of proteome research

Article

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Determination of ^{15}N-Incorporation into Plant Proteins and their Absolute Quantitation: A New Tool to Study Nitrogen Flux Dynamics and Protein Pool Sizes Elicited by Plant–Herbivore Interactions

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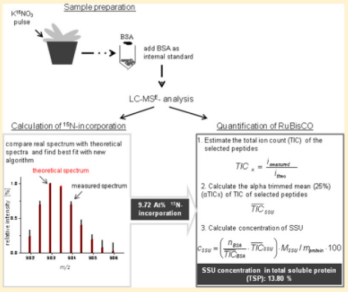
Supporting Information

ABSTRACT: Herbivory leads to changes in the allocation of nitrogen among different pools and tissues; however, a detailed quantitative analysis of these changes has been lacking. Here, we demonstrate that a mass spectrometric data-independent acquisition approach known as LC–MS^E, combined with a novel algorithm to quantify heavy atom enrichment in peptides, is able to quantify elicited changes in protein amounts and ^{15}N flux in a high throughput manner. The reliable identification/quantitation of rabbit phosphorylase b protein spiked into leaf protein extract was achieved. The linear dynamic range, reproducibility of technical and biological replicates, and differences between measured and expected ^{15}N-incorporation into the small (SSU) and large (LSU) subunits of ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO) and RuBisCO activase 2 (RCA2) of *Nicotiana attenuata* plants grown in hydroponic culture at different known concentrations of ^{15}N-labeled nitrate were used to further evaluate the procedure. The utility of the method for whole-plant studies in ecologically realistic contexts was demonstrated by using ^{15}N-pulse protocols on plants growing in soil under unknown ^{15}N-incorporation levels. Additionally, we quantified the amounts of lipoxygenase 2 (LOX2) protein, an enzyme important in antiherbivore defense responses, demonstrating that the approach allows for in-depth quantitative proteomics and ^{15}N flux analyses of the metabolic dynamics elicited during plant–herbivore interactions.



KEYWORDS: *LC–MS^E, ^{15}N-incorporation, absolute protein quantification, Nicotiana attenuata, herbivory, soil-grown plants*

INTRODUCTION

Plants fundamentally depend on inorganic nitrogen (N) for growth, reproduction, storage and defense, but N-resources in the natural environment are limited and set limits to the fitness of many plants. Thus, the production of N-containing metabolites used by plants for defense against herbivores competes with the other N pools.[1,2] A number of studies have estimated the costs of herbivore-induced defenses by tracking the N-allocation to specific secondary metabolites, and also the total N content of leaves, roots and seeds using pulse-chase labeling with ^{15}N.[3−5] However, a major source of nitrogen in plants are proteins, so that the analysis of changes in protein abundance is crucial for a better understanding of N-flux after herbivore attack. Large-scale changes in protein abundance after simulated herbivory have been measured by two-dimensional (2D) gel-electrophoresis combined with mass spectrometry (MS) for protein identification,[6,7] but the sensitivity and reproducibility of gels is limited. 2D-gels only allow for the quantitation and identification of the most abundant proteins in a complex sample[8] and is a procedure difficult to automate.[9] A high throughput quantitative method that would enable the simultaneous absolute quantitation of protein pool sizes and tracking of N-flux through the plant under ecologically relevant conditions by ^{15}N labeling has not yet been developed. The aim of our study was to develop such a method that is applicable to studying protein changes in plants elicited by herbivores.

Most modern quantitative proteomic strategies rely on stable isotope labeling and MS analysis to determine the relative quantity of a protein based on peptide abundances.[10−13] Except for one study,[14] all were performed with cell cultures, on agar plates or on hydroponically grown plants, and required a set of fully labeled and unlabeled tissues for the relative quantitation and peptide identification.[15−21] The only method available using soil-grown plants also requires a set of ^{14}N- and ^{15}N-labeled plants, which excludes the possibility of tracking nitrogen. Furthermore, the approach only allows a relative quanitification of proteins.[14] For absolute quantitation purposes, synthetically labeled standard peptides are usually added as internal standards after extraction and enzymatic digestion.[22] However, this approach requires at least one reference prototypic peptide for each protein of interest so that it is not only cost-intensive and can only be applied to a relatively low number of proteins but also

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limited to organisms that have been characterized by reliable peptide sequences.

Furthermore, it has been shown that only approximately 16% of all tryptic peptides in complex digests are accessible by data-dependent scanning modes (DDA).[23] Therefore, the absolute quantitation of proteins by a data-independent approach (DIA) was the method of choice for our study. Such a data acquisition algorithm has been developed by Silva et al. and implemented in the parallel MS^E acquisition workflow.[24] It makes use of high-resolution, accurate mass data acquired on a hybrid time-of-flight mass spectrometer that operates in alternating low energy (MS) and elevated energy (MS^E) full scan acquisition.[25] MS^E-based absolute quantitation of proteins is based on the finding that the average MS signal response of the three most intense tryptic peptides per mole of a protein is constant.[24] This fact allows for the absolute simultaneous quantitation of many proteins at once using a universal signal response factor (counts/mol) calculated in a defined dynamic range from the known amount of unlabeled protein from an evolutionary unrelated organism added prior to tryptic digestion as an internal standard. Thus, a large number of samples or conditions may be analyzed at a low cost in a high through put fashion.

Combining LC−MS^E with pulse-chase metabolic labeling with ^{15}N to track N-flux leads to unknown ^{15}N-incorporation levels in peptides. Thus, a method for the calculation of ^{15}N-incorporation levels was required for the analysis of the LC−MS^E data. Several methods for the calculation of ^{13}C and ^{15}N heavy isotope incorporation levels have been reported over the last years.[13,26−30] The general approach of these methods is to compare high-resolution peptide mass spectra with theoretical mass spectra at different incorporation levels. Except for an excel sheet published by Taubert et al.,[13] all other approaches were not suitable for our data set produced by LC−MS^E, and the excel sheet does not allow a high-throughput analysis. Therefore, we developed a novel method called MoLE (Molecule Labeling Estimator) that allows a rapid analysis of multiple spectra for determining ^{15}N-incorporation levels of peptides.

The protein quantitation approach based on LC−MS^E-data published by Silva et al.[24] relies on the intensities of the monoisotopic peak. However, increasing levels of ^{15}N-incorporation of peptides leads to a decline in the intensity of the monoisotopic peak resulting in an underestimation of the amount of the proteins of interest. We solved this problem by establishing a new approach for absolute protein quantitation based on the alpha trimmed mean of robust estimations of the total (isotope) ion count (TIC) taking the ratio of the measured to the theoretical intensity of a peak in a spectrum at its calculated ^{15}N-incorporation. This quantitation strategy is applicable to labeled proteins.

We used *Nicotiana attenuata*, an annual plant that germinates in a postfire inorganic nitrogen-rich environment in response to smoke-derived signals,[31] to validate our method. It seemed to be an ideal ecological model for protein quantitation and protein-metabolites crosstalk studies because (a) although it is a nonsequenced organism, a large number of protein sequences of closely related species (e.g. *N. tabacum* and the two important sequenced crops potato[32] and tomato[33] are available and (b) its general trade-offs between growth and defense are well-studied but a detailed analysis of these trade-offs at the protein level is still missing.

RuBisCO and RuBisCO activase 2 (RCA2) were used as example proteins because both proteins are crucial for photosynthesis and both were shown to play a role in herbivory; thus, they are ideal example proteins to study trade-offs between growth and defense.[34] Additionally, with concentrations up to 50% of total soluble protein, RuBisCO is the most abundant protein in plants and is thought to be an important N storage protein, thus playing a central role in N-metabolism. It is constructed of 16 subunits, 8 identical large ones and 8 identical small ones. RCA modulates the activity of RuBisCO by dissociating the inhibitory sugar phosphates from the active site of the enzyme independent of carbamylation and is known to influence RuBisCO amounts.[35]

We also studied a lipoxygenase (LOX) enzyme, which plays a central role in the production of signaling and defense metabolites in plants by catalyzing the dioxygenation of linolenic and linoleic acid leading to fatty acid hydroperoxides (HPs). In *Nicotiana attenuata*, NaLOX2 specifically supplies HPs for the production of so-called green leaf volatiles (GLVs) and is involved in the recycling of C_{12} derivatives,[36] while another LOX isoform, NaLOX3, catalyzes the first step in the biosynthesis of jasmonic acid (JA), a key element of plant-defense signaling.[37] GLVs and JA are induced rapidly by damage and herbivory. Interestingly, an increase in transcript levels of LOX occurs later than the maximum increase in JA and GLVs. It is thought that the initial bursts are produced by constitutive enzyme pools that are rapidly activated upon attack.[38]

Furthermore, an initial herbivore attack alters the plants ability to resist a subsequent attack. Again, it was hypothesized that inactive or active enzyme pools increase after herbivory to allow the plant to respond more rapidly to future attacks.[39] However, little is known about the enzyme pools of LOX2 and LOX3 available in the plant. Therefore, our objectives were to quantify the pools of LOX2 and LOX3 proteins in plants before and at later time-points (1−7 days) after repeated herbivory.

Thus, in this study we successfully addressed the need for a new quantitative proteomics approach enabling the determination of ^{15}N incorporation levels and protein pool sizes based on LC−MS^E data. We demonstrate that the method is applicable to soil-grown plants which received a ^{15}N pulse for tracking of changes in N-flux and elicited by simulated herbivory.

■ EXPERIMENTAL PROCEDURES

Chemicals

All chemicals were from commercial sources and were used directly without further purification.

Plant Germination, Growth Conditions and Treatment

Plants. *Nicotiana attenuata* Torr. ex. Wats (Solanaceae) seeds of an inbred line's 30th generation, whose first generation was originally collected in Utah, were sterilized and germinated on Gamborg B5 media (Duchefa) according to Kruegel et al.[40] The plants were incubated in a growth chamber at 26 °C with an 11/13 h day/night cycle. After transfer to single pots, plants were cultivated in a glasshouse at 16/8 h day/night cycle with 26− 31 °C during the day and about 20 °C during the night and 45−55% humidity. Daily conditions slightly changed depending on season.

Unlabeled Plants for Phosphorylase b Quantitation and Linear Dynamic Range Analysis. Ten days after germination, the plants were transferred to Teku pots, and 10 days later to 1 L single pots with soil. To determine the linear dynamic range, three leaves of five rosette-stage plants were pooled after the harvest. The leaves were chosen randomly.

Permanent Labeling Experiment (for the setup see also Figure S1a, Supporting Information). For the

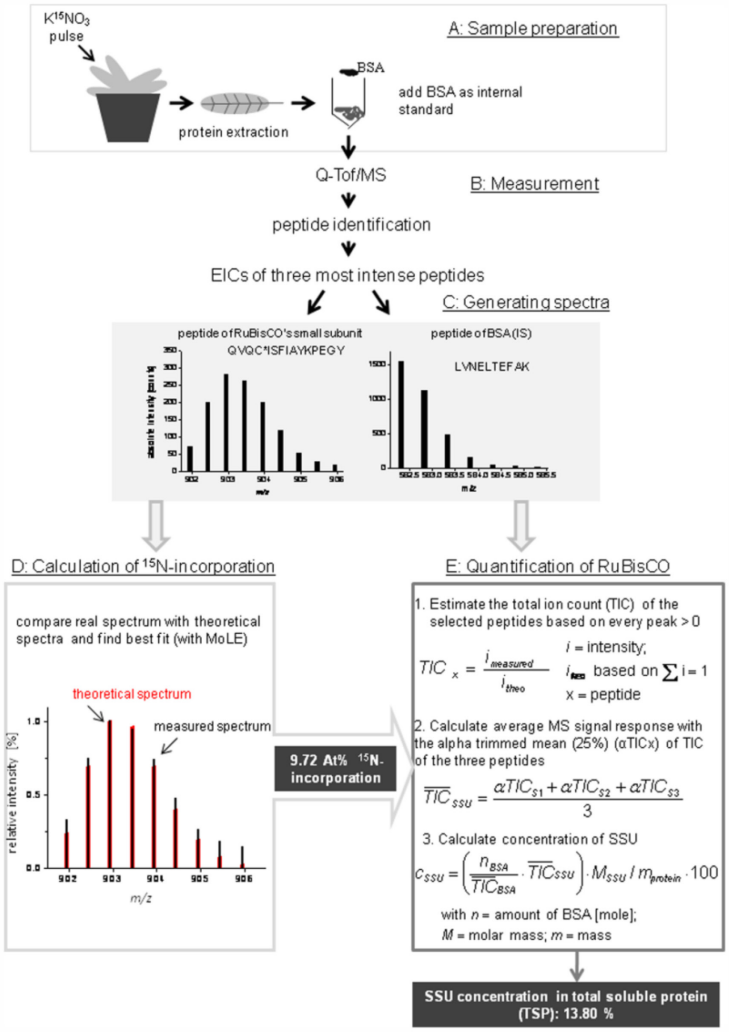

Figure 1. Experimental workflow using ribulose-1,5-bisphosphate-carboxylase/oxygenase small subunit (RuBisCO SSU) as an example. (A) Twenty-seven days after germination *Nicotiana attenuata* plants were labeled with a $K^{15}NO_3$ pulse and the oldest sink leaf was marked. Seven days later, this leaf was harvested. After the extraction of total soluble protein, bovine serum albumin (BSA) was added as an internal standard before tryptic digestion. (B) Samples were analyzed on a LC−MS^E system. The three most intense peptides of RuBisCO SSU and of BSA (Table 1) were identified by their retention time (RT), mass and charge. Extracted ion chromatograms (EIC) of each peptide were generated. (C) Spectrum of 5 scans around the maximal intensity of the three selected peptides of RuBisCO SSU was created and used for ^{15}N-incorporation calculation. For quantitation, a spectrum of the scan with the highest absolute intensity was produced for the 3 selected peptides of SSU and of BSA. (D) ^{15}N-Incorporation was determined with a novel algorithm (MoLE) by comparing the pattern of the spectrum of each peptide to theoretical patterns in a library. (E) For quantitation, the total ion count (TIC) of all peaks of a spectrum was estimated by taking the ratio of the measured intensity to the theoretical intensity (derived from the theoretical spectrum of ^{15}N-incorporation) (1). On the basis of all individual TICs of a peptide, the alpha trimmed mean (25%) was calculated (2). The TIC alpha trimmed means of the three peptides of RuBisCO SSU and the internal standard BSA were averaged and used to calculate the percentage of RuBisCO SSU in total soluble protein (TSP, determined by the Bradford method) (3).

permanent labeling experiment, the plants were transferred after 12 days into 50 mL hydroponic culture single pots containing different concentrations of ^{15}N-labeled $Ca(NO_3)_2$ (0, 1, 5, 10, 50, 100 At%), and 10 days later to 1 L hydroponic culture pots with the same $^{15}NO_3^-$ concentrations in the form of KNO_3 and kept in the glasshouse. For the first 12 days in hydroponic culture, the plants were fertilized with 0.210 g Flory P/K (Euflor, Munich, Germany), 0.0536 g $MgSO_4$, 0.455 mL Fe·diethylenetriamine-pentaacetic acid (Fe·DTPA) solution (2.78 g $FeSO_4 \cdot 7H_2O$ and 3.93 g $C_{14}H_{23}N_3O_{10}$ per L) and 2 mmol $Ca(NO_3)_2$ per L. After transfer to 1 L single pots, they were fertilized with 0.123 g $MgSO_4 \cdot 7H_2O$, 0.129 g $CaSO_4 \cdot H_2O$, 0.048 g K_2HPO_4, 0.031 g KH_2PO_4, 0.5 mL micronutrient solution (2.533 g H_3BO_3, 1.634 g $MnSO_4 \cdot 2H_2O$, 0.151 g Na_2MoO_4 $2H_2O$, 0.440 g $ZnSO_4 \cdot 7H_2O$,

0.080 g $CuSO_4 \cdot 5H_2O$, 0.020 g $CoCl_2 \cdot 6H_2O$ per L H_2O), 0.5 mL Fe·DTPA and 1 mmol KNO_3 per L until harvest. This fertilization regime resulted in 0.37, 1.35, 5.28, 10.19, 49.49, and 98.16 At% in the plants if the natural abundance of ^{15}N is considered. Once per week, all pots received 1 mmol KNO_3 per L with the same concentrations of $^{15}NO_3^-$ they received when still in 50 mL pots, and the pots were filled up to 1 L with water. Labeled media were prepared by substituting $^{14}NO_3^-$ for $^{15}NO_3^-$ at appropriate concentrations without consideration of the natural abundance of ^{15}N. The plants were harvested 10 days after transfer to 1 L pots. All analyses were carried out with −2 sink leaves at the time of harvest.[41]

Pulse Labeling Experiments (see also Figure 1 for the experimental workflow and Figure S1, Supporting Information, for the setup). For the pulse labeling experiment 27-day-old plants were labeled with 5.1 mg nitrogen in the form of $K^{15}NO_3$ and the oldest sink (−1) leaf was marked. For a kinetic analysis, the oldest sink leaf at the time of labeling was harvested 3, 4, 6, 7, and 10 days after the $K^{15}NO_3$-pulse. Three reference plants received the same amount of unlabeled fertilizer.

To study the role of LOX2 and LOX3 after repeated herbivory, a transgenic line which has reduced expression of NaLOX3 (ir*LOX3*; A-03-562-2,[36]) and a co silencing of NaLOX2 was used in addition to *N. attenuata* WT and germinated as described above for a further validation of our approach. Three days after pulse labeling the oldest sink leaf, youngest source leaf and transition leaf (0) at the time-point of labeling plants were wounded (W) with a pattern wheel and treated with 10 μL 1:5 diluted *Manduca sexta* oral secretion (OS) (W+OS) on three consecutive days. The time-shift between labeling and treatment was used because in a preliminary experiment ^{15}N incorporation in leaves reached its maximum three days after labeling. At this time-point there were not significant differences in amino acid residues ^{15}N-labeling pattern (calculated from CID-fragmentation spectra of individual peptides, data not shown). Untreated plants were used for control. The oldest sink leaves were harvested 0, 1, 3, 4, and 7 days after the first treatment.

Total Protein Extraction and Quantitation

To extract the soluble proteins, the leaf tissue was homogenized by GenoGrinder2000 (SpexCertiPrep, Middlesex, U.K.) in 200 μL 0.1 M Tris-HCl (pH 7.6) per 100 mg leaf tissue. The tissue was completely suspended by Vortex and the samples shaken for 2 min. After centrifugation for 25 min at 16100× *g* at 4 °C, the supernatant was transferred to a fresh tube. The protein concentration was measured using a Bio-Rad protein assay kit (Biorad, Hercules, CA) with bovine serum albumin (BSA) as standard.

Sample Preparation for LC−MS^E Analysis

Sample Preparation for Phosphorylase b Quantitation. Five micrograms of total soluble protein and 0.498 μg (7.5 pmol) BSA as internal standard were combined with different amounts (3 samples per concentration: 5, 10, 20, 40, 50 pmol) of phosphorylase b from rabbit (Sigma Aldrich, Germany) in a total volume of 60 μL 0.1 M Tris-HCl-buffer (pH 7.6). Phosphorylase b (1 pmol/μL) dissolved in 25 mM ammonium bicarbonate buffer was used as stock solution. The samples were further prepared as described below.

Preparation of All Other Samples. Five micrograms of total soluble protein and 0.334 μg (5 pmol) BSA as internal standard were combined in a total volume of 20 μL 0.1 M Tris-HCl-buffer.

Reduction, Alkylation and Digestion of All Samples. The samples were reduced by the addition of an equal volume of 20 mM DTT (final concentration of DTT: 10 mM). The samples were incubated at 80 °C for 20 min, cooled to room temperature (RT) and 1 volume of 55 mM iodoacetamide was added to alkylate the reduced cysteines by incubation at 4 °C in the dark for 2 h. The proteins were then precipitated by adding 0.25 times the volumes of 50% TCA (final concentration 10% w/v) at 4 °C for 45 min. After centrifugation for 15 min at 4 °C with 16100× *g*, the precipitates were washed with 80% acetone, centrifuged again at 4 °C with 16100× *g* for 5 min and dried afterward. Proteolysis was initiated by dissolving the dry protein pellet in 25 μL of 50 mM ammonium bicarbonate containing 65 ng of sequencing grade porcine trypsin (Promega, Madison, WI) to each sample and digestion was carried out overnight at 37 °C. After being vacuum-dried to remove any remaining liquid and ammonium bicarbonate, the dried and digested proteins were kept at −20 °C. Prior to further analysis, the samples were redissolved in 0.1% formic acid containing 3% acetonitrile.

Chromatographic and Mass Spectrometry Conditions

Samples were separated using a nanoAcquity nanoUPLC system (Waters, Manchester, UK). A mobile phase of 0.1% aqueous formic acid was used to concentrate and desalt the sample on a Symmetry C18 trap-column (20 × 0.18 mm, 5 μm particle size) at a flow rate of 15 μL min^{-1}. Subsequently, peptides were eluted onto a nanoAcquity C18 column (200 mm × 75 μm ID, C18 BEH 130 material, 1.7 μm particle size) using an increasing acetonitrile gradient in 0.1% aqueous formic acid at a flow rate of 0.350 μL min^{-1}. Buffers A (0.1% FA in H_2O) and B (100% MeCN, 0.1% FA) were linearly mixed in a gradient from 1% to 45% phase B over 60 min, increased to 95% B over 5 min, held at 95% B for 5 min and decreased to 1% B over 1 min. The analytical column was immediately re-equilibrated for 9 min.

The eluted peptides were transferred to the nanoelectrospray source of a Synapt HDMS tandem mass spectrometer (Waters, Manchester, U.K.) equipped with a metal-coated nanoelectrospray tip (Picotip, 50 × 0.36 mm, 10 μm internal diameter, New Objective, Woburn, MA). The source temperature was set to 80 °C, cone gas flow 20 L/h, and the nanoelectrospray voltage was 3.2 kV. For all measurements, the mass spectrometer was operated in V-mode with a resolving power of at least 10000 fwhm. All analyses were performed in positive ESI mode. A 650 fmol/μL human Glu-fibrinopeptide B in 0.1% formic acid/acetonitrile (1:1 v/v) was infused at a flow rate of 0.5 μL min^{-1} through the reference NanoLockSpray source every 30 s to compensate for mass shifts in MS and MS/MS fragmentation mode.

LC−MS data were collected using alternating low energy (MS) and elevated energy (MS^E) modes of acquisition over 1.5 s intervals in the range m/z 50−1700 with an interscan delay of 0.2 s. In low energy mode, data were collected at constant collision energy of 4 eV set on the trap T-wave device and ramped during scan from 15 to 40 eV in elevated MS^E mode. The chromatographic and mass spectrometry performances of the system were regularly checked using tryptically digested BSA and *N. attenuata* wild type samples.

Data Processing and Protein Identification

The LC−MS^E data of unlabeled samples from a continuum were collected using MassLynx v4.1 software and processed by ProteinLynx Global Server Browser v.2.4 software (Waters, Manchester, U.K.) under baseline subtraction, smoothing, deisotoping, lockmass-correction and alignment according to

Table 1. Peptides of Ribulose-1,5-bisphosphate-carboxylase/oxygenases (RuBisCO) Large (L) and Small (S) Subunit and RuBisCO Activase 2 (RCA2) (R) from *Nicotiana attenuata* and of BSA (B) Used for Absolute Protein Quantitation and for Calculation of the ^{15}N-Incorporation of Hydroponically-grown Plants[a]

no	calc. $[MH]^+$	exp. $[MH]^+$	Δ ppm[b]	Rt [min]	Rt RSD [%]	sequence	sumformula
L1	1021.5313	1021.5313	0.0	41.05	0.7	DTDILAAFR	$C_{45}H_{72}N_{12}O_{15}$
L2	1261.7150	1261.7148	0.2	51.09	0.4	DITLGFVDLLR	$C_{58}H_{96}N_{14}O_{17}$
L3	1579.8367	1579.8376	0.6	47.94	0.5	EIVFNFAAVDVLDK	$C_{74}H_{114}N_{16}O_{22}$
S1	2069.0437	2069.0519	4.0	45.13	0.5	YETLSYLPDLSEEQLLR	$C_{93}H_{145}N_{21}O_{32}$
S2	1802.8781	1802.8848	3.7	39.66	1.1	QVQC*ISFIAYKPEGY	$C_{83}H_{123}N_{19}O_{24}S_1$
S3	933.5152	933.5128	2.7	33.78	2.4	IIGFDNVR	$C_{42}H_{68}N_{12}O_{12}$
B1	1163.6306	1163.6395	7.7	35.98	1.6	LVNELTEFAK	$C_{53}H_{86}N_{12}O_{17}$
B2	1479.7954	1479.7966	0.8	41.37	2.5	LGEYGFQNALIVR	$C_{68}H_{106}N_{12}O_{19}$
B3	1567.7427	1567.7454	1.7	47.87	1.0	DAFLGSFLYEYSR	$C_{74}H_{102}N_{16}O_{22}$
R1	1214.6263	1214.6298	2.9	28.17	1.0	TDNVPEEAVIK	$C_{52}H_{87}N_{13}O_{20}$
R2	1706.7980	1706.8067	5.1	36.37	2.7	GLVQDFSDDQQDIAR	$C_{71}H_{111}N_{21}O_{28}$
R3	1332.6794	1332.6839	3.4	33.61	1.8	WVSGTGIEAIGDK	$C_{59}H_{93}N_{15}O_{20}$

[a]Three most intense peptides were taken, except from RuBisCO LSU where the 4th, 5th and 7th most intense peptides were used. The table includes the average mass and retention time (Rt) calculated from triplicate analysis. Carbamido methylated methionine is denoted as C*. [b]Δ ppm = $10^6(M_{tn} - M_{exp})M_{tn}^{-1}$

the ion accounting algorithm.[42] The processed data were searched against the Uniprot "*Nicotiana tabacum*" subdatabase (downloaded 6 January 2009) to which sequences from BSA, trypsin and phosphorylase b from rabbit were appended. The search parameters were set as follows: mass accuracy of 10 ppm for precursor ions and 15 ppm for product ions, a minimum of 2 peptide matches per protein, with a minimum of 3 consecutive fragment ions each. The maximum false positive rate (FDR) against the randomized database was set to 2%. Further settings were one possible missed tryptic cleavage site, a fixed carboxymethylation of cysteines, variable deamidation of asparagine/glutamines and variable oxidation of methionines. On the basis of our database search of unlabeled wild type samples, on average 90 proteins were found in the protein extracts, and about half of those could be identified.

N. attenuata's RuBisCO LSU consists of 477 amino acids and has a mass of 52.90 kDa, RuBisCO SSU of 180 with a mass of 20.35 kDa and RCA2 of 439 with a mass of 48.28 kDa, respectively.

Data Processing of ^{15}N-Labeled Samples

Extracted ion chromatograms (EIC) of the light form of three peptides of the protein of interest (phosphorylase b, BSA, RuBisCOactivase 2 (RCA2), RuBisCO large (LSU) and small subunit (SSU) and lipoxygenase 2 (LOX2) (Tables 1, S2, S3, Supporting Information) were generated from the full scan mass spectra based on their m/z of the identified peptides and their retention time. The peptides were chosen based on three criteria: (1) most intense peptides, (2) peptides that showed analytical reproducibility, and (3) no overlap in spectra. As the most intense peptides of the proteins of interest varied between experiments, they were determined individually for each experiment by peptide lists generated with PLGS v2.4 of unlabeled samples (Tables 1, S2, S3). For ^{15}N-incorporation estimations, average spectra were generated from 5 scans at the top of the chromatographic peak, and for quantitation, one spectrum was generated of the scan at the top. They were baseline subtracted, smoothed and centroided manually in MassLynx v4.1 software. The following settings were used: Baseline subtraction, polynomial order 20, below curve 5%, tolerance 0.010 Da; smoothing, Savitzky-Golay with smooth window of 3 channels, applied twice to each spectrum; Centroid calculation, minimum peak width at half height of 4 channels and centering at peak width at 35% from the top. Subsequently, the masses were lockmass-corrected by the same software. For lockmass, a correction factor was calculated based on the ratio of the measured mass to the theoretical mass of the lockmass substance 785.8426 Da. To determine the measured mass, a spectrum of the calibration trace was created in a mass window of 30 min. During this period of time, all intense peptides were detected. The correction factor was applied as follows:

$$\textbf{correction factor} = \frac{\text{theoretical mass}_{785.8426}}{\text{measured mass}_{785.8426}} \quad (1.1)$$

The measured monoisotopic masses were multiplied with this factor.

Quantitation of Phosphorylase b from Rabbit, RuBisCO, RCA2, and LOX2

The absolute quantity of each protein of interest (calc_c) (phosphorylase b, RuBisCO SSU, RuBisCO LSU, RCA2, LOX2) was calculated based on the relation between the average of the robust estimations of total ion count (TIC) of the three most intense peptides of internal standard (IS, BSA) and the three most intense peptides of the protein of interest (Table 1, S2, S3, Supporting Information): First the TIC of each peptide was estimated based on the measured intensity ($ix_{measured}$) of every peak (x) > 0 of the spectrum and its ratio to its theoretical intensity (ix_{theo}) at its individual ^{15}N-incorporation level. The theoretical intensity is the relative intensity of a peak based on a theoretical spectrum at its ^{15}N-incorporation. Then the alpha trimmed means of TIC of the selected peptides (px, p = kind of protein) (αTIC_{px}), calculated based on all robust TIC estimations of this peptide, were averaged to get the average total ion count ($\overline{TIC}$) (Figure 1). We used the alpha trimmed mean to minimize the influence of outliers, which often result in inaccurate results from nonrobust estimators such as the mean, or simply using the sum of peak intensities as TIC. Finally, the average total ion count estimates of all charge states of a peptide were added. The calculation is shown for RuBisCO SSU (RUB−SSU=S) as example:

$$TIC_x = \frac{ix_{measured}}{ix_{theo}} \text{ with } \sum ix_{theo,i} = 1 \quad (1.2)$$

$$\overline{\mathrm{TIC}}_{\mathrm{RUB-SSU}} = \frac{\alpha\mathrm{TIC}_{S1} + \alpha\mathrm{TIC}_{S2} + \alpha\mathrm{TIC}_{S3}}{3} \quad (1.3)$$

$$\mathrm{calcc}_{\mathrm{RUB-SSU}} = \left(\frac{n_{\mathrm{BSA}}}{\overline{\mathrm{TIC}}_{\mathrm{BSA}}} \times \overline{\mathrm{TIC}}_{\mathrm{RUB-SSU}}\right) \times M_{\mathrm{RUB-SSU}}/m_{\mathrm{protein}} \times 100 \quad (1.4)$$

With n = amount of BSA (mole), M = molar mass of protein of interest, m = mass of total soluble protein on column.

MoLE: Molecule Labeling Estimator

To estimate the ^{15}N-incorporation, we developed a new method implemented in the program MoLE "Molecule Labeling Estimator". It requires a list of masses M_0;...;M_K with intensities f_0; ....;f_K normalized such that $\sum_i f_i = 1$ as input. The isotope pattern of the sample molecule is extracted from the mass spectrum based on the sum formula of the molecule (peptide) and its charge. The basic concept of the algorithm is to simulate the isotope pattern of the sample molecule for different incorporations and to match those with the isotope pattern of the measured sample. The predicted isotope distribution is based on all natural-abundance isotopes, except the element that is selected to be enriched. The simulation of the isotope pattern with masses m_0; .....;m_K and intensities p_0;.....;p_K is computed by deconvoluting the isotope distributions of the individual elements of the molecule.[43] All possible incorporation rates between 0 and 100% are tested in steps size 10. After the best initial scoring incorporation ratio r has been determined, the second phase of the algorithm is started, where the ratio is varied between $r - 10$ and $r + 10$ with step size 1. In subsequent phases, additional digits of the ratios can be determined.

To score each incorporation ratio, the statistical model introduced in Böcker et al.[43] is used. This score is based on Bayesian statistics and the maximum likelihood framework. The likelihood is computed to observe the data $\mathcal{D}$ under the model $\mathcal{M}$ of a particular incorporation ratio and the background model $\mathcal{B}$ that one of the incorporation ratios is correct:

$$\mathbb{P}(\mathcal{D}|\mathcal{M}, \mathcal{B}) = \prod_j \mathbb{P}(M_j|m_j) \prod_j \mathbb{P}(f_j|p_j) \quad (1.5)$$

Here the independence of the individual probabilities is assumed, where $\mathbb{P}(M_j|m_j)$ is the probability of observing a peak with mass M_j when the true mass of the ion is m_j and $\mathbb{P}(f_j|p_j)$ is the probability of observing a peak with intensity f_j when the true intensity of the ion is p_j.

The ^{15}N-incorporations were calculated based on the more relevant ^{13}C isotope abundance of 1.096%, instead of the carbonate specific abundance of 1.112%, since this appears to be more appropriate in biological studies.[44]

The program also allows scoring incorporation ratios using mass differences instead of masses; therefore, we could calibrate using the monoisotopic peak; or to ignore masses, so that the score is based solely on intensities. As we have observed that peaks of low intensity often show high variations in the measured intensity, the program offers the option to remove all such peaks from the analysis. Furthermore, to counter the effect of an imprecise baseline correction, there is an additional option to subtract the median intensity of the removed peaks from the intensity of each of the remaining peaks. To account for modifications such as carbamidomethylation, carboxylation and hydroxylation MoLE offers the option of entering the molecular formula of the modification where the enrichment is not taken into account and the natural ^{15}N/^{14}N ratio is used.

The program is freely available and can be downloaded from http://bio.informatik.uni-jena.de/software/mole/.

Calculation of ^{15}N-Incorporation

The processed spectra of the three chosen peptides of the protein of interest (RCA2, RuBisCO SSU and LSU, LOX2) (Table 1, S2, S3, Figure S2, Supporting Information) were used for the calculation of the ^{15}N-incorporation of the protein. The program MoLE (see above) and the script published by Taubert et al.[13] were used to calculate the ^{15}N-incorporation for each peptide. The average ^{15}N-incorporation of the three peptides was then defined as the overall ^{15}N-incorporation of the protein.

For the calculation of the ^{15}N-incorporation by MoLE, we used intensities only, since the results for our data set were not improved by using additional settings. First, all peaks of the averaged spectra with relative intensities between 0 and 2% were removed, and the median of the intensities of the removed mass peaks was subtracted from the intensities of the remaining peaks. This step was necessary to adjust the unequal impact of baseline correction on peaks of different intensities. Furthermore, peaks below 3 and 7% relative intensity were not scored, because small peaks were often less precise. This adjustment was applied so that unlabeled samples were close to the natural ^{15}N abundance of 0.3677 At%. If peptides had modifications which were added to the peptide during the sample preparation, they were defined as naturally labeled parts of the molecular formula. For unlabeled samples, ^{15}N abundance was set to 0.3677 At%.

Sample Preparation for Isotope Ratio Mass Spectrometry

To reconfirm the ^{15}N-labeling calculated from LC−MS^E data, the labeling of total soluble protein was also analyzed by an elemental analyzer−continuous flow−isotope ratio mass spectrometry (EA−CF−IRMS). Three hundred micrograms of dissolved protein was filled into 40 μL tin capsules for liquid samples (IVA Analysentechnik, Meerbusch, Germany) and dried before analysis. To accommodate the high sensitivity of the IRMS, samples were diluted to a final labeling of about 1 At% ^{15}N by adding a 5 μg/μL solution of unlabeled BSA to the tin capsules.

For the determination of ^{15}N-incorporation in leaves, the plant tissue was dried at 60 °C for 48 h and homogenized before analysis. About 0.125 ± 20% mg of the homogenized and dried plant material was put into 40 μL tin capsules together with 0.8325 ± 20% mg of working standard (acetanilide) to ensure that the labeling did not exceed about 1 At% ^{15}N (and thus avoid detector saturation). The exact plant sample weights were measured and used for calculation of ^{15}N abundance. Three technical replicates for each sample were analyzed.

Isotope Ratio Mass Spectrometry Analysis

The IRMS analysis and the calculation of ^{15}N-incorporation were carried out as described by Meldau et al.[45]

Evaluation of ^{15}N-Incorporation over Time

The increase in labeling over time was characterized by a sigmoidal regression analysis using the Gompertz function with the software Origin 8G (Originlab, Northampton, MA). Based on the function:

$$y = ae^{-e^{(-k\cdot(t-t_i))}} \quad (1.6)$$

with a representing the horizontal asymptote, t_i the time-point of inflection and k a constant related to the increase in labeling. We

calculated the maximal slope s as an indicator of ^{15}N incorporation per day as follows:

$$s = \frac{a \cdot k}{e} \quad (1.7)$$

RESULTS AND DISCUSSION

The aim of this study was to develop a new strategy for the absolute quantitation of proteins with unknown ^{15}N-incorporation levels which would enable us to study changes in the protein pools and ^{15}N flux after herbivore attack of plants in detail. Robustness and reliability of the strategy was analyzed thoroughly before applying it to soil-grown elicited plants with simulated herbivory.

Quantitation Strategy

Our quantitation strategy is based on Silva et al.[24] who established a data-independent approach (DIA) for absolute quantitation of proteins in complex leaf samples, also known as LC−MS^E. The approach uses a universal signal response factor of a single internal standard for protein quantitation. To calculate the universal signal response factor the authors summed the intensity of the deisotoped monoisotopic peak over the whole chromatographic peak of all charge stages for each of the three most intense peptides of the internal standard. The averaged intensity of those three peptides divided by the quantity of the internal standard was defined as the universal signal response factor. The average signal response of the protein of interest was calculated as described for the internal standard and used with the universal signal response factor for quantification. This method was not suitable for our samples because plants were labeled with ^{15}N, and increasing ^{15}N-incorporation decreases the intensity of the monoisotopic peak as a function of the sum formula of the peptide and its labeling. Therefore, we developed a new strategy and calculated the universal signal response factor based on the robust estimations of total ion count (TIC) of the three most intense peptides as shown in Figure 1E (see also Experimental Procedures, eqs 1.2−1.4). In brief, we defined the signal response of a peptide as alpha trimmed mean of TIC. The alpha trimmed mean of TIC was calculated from a set of robust TIC estimations which are based on the ratio of measured intensity and theoretical intensity of every peak of a spectrum >0 at the top of the chromatographic peak. As defined above, the theoretical intensity is the relative intensity of a peak based on a theoretical spectrum at its specific ^{15}N-incorporation level. On the basis of the alpha trimmed mean of TIC of the three most intense peptides of the internal standard, its average signal response was determined. By dividing it through the quantity of internal standard, we obtained the universal signal response factor. The average signal response of the protein of interest was calculated in the same way and quantified based on the universal signal response factor of the internal standard. This strategy for protein quantitation is independent of the isotopic distribution and the sum formula of a peptide, so that it is applicable to all labeled proteins.

Validation of the Quantitation Approach with Phosphorylase b

To test the applicability of here developed LC−MS^E-based quantitation strategy in our complex leaf samples, we added different amounts of phosphorylase b from rabbit to internal standard-spiked unlabeled total soluble protein extracts. We chose BSA as internal standard because it is a midsized protein (585 amino acids) similar to the studied ones and cannot be synthesized by plants. The tryptic digests of these extracts were analyzed using an alternate scanning mode of data acquisition (LC−MS^E) on a Q-Tof mass spectrometer. Based on the LC−MS^E data we generated peptide lists with PLGS v2.4. Peptides corresponding to the studied proteins were identified by searching with an MS-Blast homology-based searching algorithm[46] against a subdatabase downloaded from Uniprot (see Material and Methods). We used the peptide list to determine the three most intense peptides of BSA and phosphorylase b (Table S2, Supporting Information). For peptide selection, in addition to the signal intensity, we also used further criteria. Some of the peptides were not detectable in all analyzed samples due to isotopic overlap in spectra with other peptides. Therefore, we selected only those peptides for quantitation that could be reproducibly detected and did not overlap with other peptides.

Phosphorylase b was quantified based on its average signal responses and the universal signal response factor of BSA (Figure 1E, Figure 2) as described above. We conducted triple measurements of each sample to analyze the technical reproducibility. Phosphorylase b could be reliably quantified up to an amount of 300 fmol on the column with a maximal relative deviation of 20.18%, which is equivalent to 38.8% in total soluble protein (TSP). Samples with concentrations lower than 300 fmol could be measured with a reproducibility of 10%. For higher injected amounts (up to 750 fmol on the column; ∼97% in TSP) the amounts of proteins determined was lower than expected and the deviation of determination increased significantly (34.27%). It is probably related to an oversaturation of the ion detector in the used instrumentation (see later for detailed explanation). Silva et al.[24] showed a deviation in a similar range (15%) after applying six exogenous proteins in varying concentrations to samples of the human serum. The data demonstrate that a reliable quantitation of proteins in our complex leaf extracts is

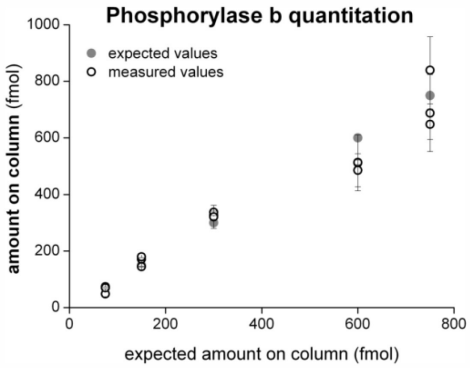

Figure 2. Calculated and expected amounts of phosphorylase b after addition of different known amounts to complex leaf samples. Five μg of total soluble protein (TSP) of *Nicotiana attenuata* (three independent samples per concentration) were spiked with different amounts of phosphorylase b from rabbit muscle and a standard amount of BSA as internal standard prior sample preparation for LC−MS^E measurement (see Figure 1). The protein quantity of phoshorylase b was calculated based on the universal response factor of BSA and the robust estimations of the total ion counts (TIC) of the three most intense peptides of phosphorylase b (Table S1, Supporting Information). Each sample was measured three times. The variability of technical replicates of each sample is indicated by the error bars.

possible using LC−MS^E data and the estimation of the universal response factor based on TIC.

Peptide Identification of RuBisCO LSU and SSU and RCA2 of *N. attenuata*

To further validate the applicability of our approach for leaf proteins, two important proteins of plants, RuBisCO and RuBisCO activase (RCA), which are regulated by herbivory[34] and involved in regulation of nitrogen dynamics in leaves[47,48] were chosen as model proteins for quantitation and nitrogen flux studies. As *N. attenuata* is an a nonsequenced organism, and the protein sequences of *N. attenuata* were not available at the beginning of our study, we used peptide sequences of *N. tabacum* and *N. sylvestris* for peptide identification, because the phylogenetic distance of *N. attenuata* to *N. tabacum* and *N. sylvestris* is close.[49] In fact, the very recently obtained complete mRNA sequences of *N. attenuata* in our laboratory showed for RuBisCO LSU (JF419563) 99% identity to *N. tabacum* (NP_054507), and for SSU (JF419564) 96% identity to *N. sylvestris* (P22433) after translation to the protein sequence. From three RCA isoforms occurring in *N. attenuata* (JF419565, JF419566, JF419567), the most abundant isoform, RCA2, showed a 99% identity to *N. tabacum* RCA2 (Q40565). These data demonstrate that our approach also allows absolute protein quantitation of nonsequenced organisms, as long as a close related sequenced species are available whose genome can be used for peptide identification.

We found a concentration-dependent protein sequence coverage from 23.9 to 48.2% for RuBisCO LSU, 29.3 to 59.7% for RuBisCO SSU and 18.2 to 58.5% for RCA2 (data not shown). Accurate measurements with a relative standard deviation (RSD) of retention time less than 2.7% and a highest mass precision error of 5.1 ppm (expect for one peptide, Table 1 and S3) allowed a precise peptide identification. Independent experiments showed similar intense peptides, while order of the most intense peptides varied (data not shown), so that different peptides for quantitation were selected for the different experiments (Table 1 and S3). We further observed that LC−MS^E data for RuBisCO LSU resulted in peptides whose intensity was significantly higher compared to the internal standard BSA and other model proteins. LSU has many basic sites which might result in a better ionization and consequently higher intensities of these peptides. To avoid detector oversaturation that might result in (a) incorrect calculations of ^{15}N-incorporation levels and (b) a quantitation error, we chose peptides with a maximum intensity lower than 2000 counts for RuBisCO LSU quantitation. This may lead to a small underestimation of the average signal response of RuBisCO LSU, but for all other evaluated proteins, the data-independent acquisition approach was fully applicable.

Determination of the Linear Dynamic Range (LDR)

For the sample analysis we used a microchannel plate detector of ions (MCP) equipped with a time-to-digital data converter. These detectors provide high sensitivity, high speed (in picoseconds) and low background noise, but MCPs have certain limitations due to their inability to count precisely if more than one ion simultaneously hits the detector. This lowers MCP linear dynamic ranges at high intensities of ions (linearity approximately 2.5 orders of magnitude) compared to other types of detectors, such as photomultipliers in tandem quadrupoles or the novel hybrid detectors.[50]

Our complex leaf samples contain unknown amounts of RuBisCO LSU and SSU and RCA2. To determine the linear dynamic range (LDR) of the detector for quantitation and the optimal amount of protein required for their LC−MS^E analyses, different amounts of total protein were analyzed. The average MS signal defined as the average of alpha trimmed mean of TIC of the three chosen peptides (Table 1) of RuBisCO LSU, RuBisCO SSU and RCA2 (example proteins) and BSA (internal standard) were calculated. The total amount of protein (total soluble protein (TSP) on column) injected ranged from 15.6 to 500 ng. Figure 3 represents the relationship between the amount of protein loaded onto the column and the average signal response: BSA was linear in the full range tested with a correlation coefficient of 0.9911, whereas RuBiSCO SSU and LSU ranged between 15.6 to 125 ng (R^2 SSU: 0.9928 and LSU: 0.9750) RCA2 from 15.6 to 250 ng (R^2 RCA2: 0.9911) (Figure 3), giving an average linear dynamic range of about 1.5 orders of magnitude.

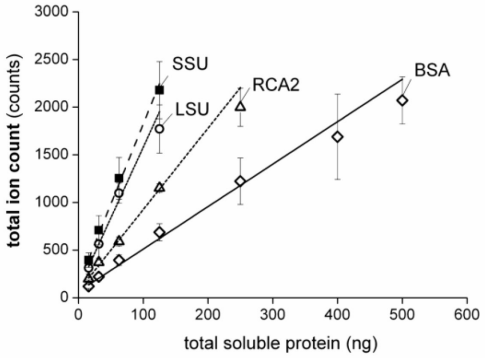

Figure 3. Linear dynamic range of LC−MS^E measurements. Signal response for RuBisCO large (LSU) and small subunit (SSU), RuBisCO activase 2 (RCA2) (R) and BSA plotted against the amount of the analyzed total soluble protein (TSP). The averaged total ion count (mean ± SE, n = 3) for each protein was calculated using selected peptides (Table 1, L1−3; S1−3; R1−3; B1−3). Data were analyzed by a linear regression (R^2: LSU = 0.9750; SSU = 0.9928; RCA2 = 0.9911; BSA = 0.9741).

Most proteins in the sample are less abundant than RuBisCO SSU and LSU, so that initially an amount of 250 ng of TSP was chosen for analysis. However, for our three studied proteins it was sufficient to use 75 ng as optimal protein amount for the LC−MS^E analysis in the given dynamic range of protein concentration.

Labeled Peptide Identification and Determination of ^{15}N-Incorporation

The aim of our study is not only the absolute quantitation of proteins, but to study N-flux in plants after herbivore attack which requires labeling of plants with ^{15}N. However, ^{15}N-incorporation into proteins causes shifts in the isotope pattern of mass spectra of peptides used for protein identification and quantification. Thus, two additional challenges had to be faced: Identification of labeled peptides and determination of ^{15}N-incorporation of each peptide, which is a prerequisite for protein quantification.

Peptide identification algorithms applied in conventional database searching software are well established for peptides of natural isotopic distribution, but metabolic ^{15}N-labeling of plants never reaches 100% and results in ubiquitous labeling which dramatically reduces the efficiency of peptide identification by

database searching.[51] Therefore, here, we harvested for each experiment three untreated plants per time point, extracted proteins and generated peptide lists with PLGS v2.4 as described above. The peptide lists were used to identify peptides for quantitation (Table 1, Table S3, Supporting Information). Based on this information, peptides of the labeled samples were identified manually according to their identical retention times and identified m/z values of the unlabeled peptide to demonstrate the proof of principle. Upon software modifications, such identification/quantitation should be possible in an automated manner.

For our protein quantitation approach, we used the robust estimation of TIC which requires the theoretical intensity of each peak of a spectrum (see above and Experimental Procedures). As the spectrum is influenced by the level of ^{15}N labeling the individual ^{15}N-incorporation had to be determined for each peptide. To estimate the isotope enrichment of labeled peptides, we developed a program, called MoLE "Molecule Labeling Estimator", which simulates different ^{15}N-incorporations of a sample molecule and matches them with the isotope pattern of the measured samples using the best probable fit to determine the ^{15}N-incorporation.

Previous approaches used the mean square error[13,26,29] or the Pearson correlation coefficient[27] of peak intensities to score incorporation ratios. Jehmlich et al.[52] proposes to use the accurate mass of the most intense peak to determine this ratio. Pan et al.[28] combine peptide identification and determination of ^{15}N-incorporation in a single analytical step. MoLE uses both intensities and mass shifts, integrated into a single probabilistic model of the data. This statistical model was originally introduced by Böcker et al.[43] for molecular formula identification using isotope patterns. It is based on a Bayesian probabilistic model in combination with a maximum likelihood estimation which has proven to be beneficial in numerous areas of data analysis. Unlike the approach of Böcker et al.,[43] MoLE uses additional parameters for peak list processing and additional information for the molecular formula, to estimate heavy isotope enrichments of molecules. In addition, MoLE comes with an easy-to-use graphical user interface, and allows batch processing of mass spectra. It could be easily modified to be applicable for other heavy nuclide isotopes (^{2}H, ^{18}O, ^{13}C) enrichment calculations.

To assess the accuracy of ^{15}N level calculations, plants with different constant ^{15}N-labeling from natural abundance to almost 100 At% were cultured, and the ^{15}N-incorporations of RuBisCO LSU's and SSU's peptides (Table S1, Supporting Information) were determined for 5 biological replicates. The difference from the expected ^{15}N-incorporation was for all peptides and labeling levels, except two peptides at 49.49 At%, less than 1 At% (Figure 4). To validate the program MoLE, the ^{15}N-incorporation for LSU and SSU was also calculated with a previously published macro Excel sheet.[13] The values obtained with the macro Excel sheet were similar to those obtained with MoLE and showed similar deviations from the expected values (Figure S3, Table S1). However, MoLE has the advantage that peaks are identified automatically and the ^{15}N-incorporation of multiple spectra can be calculated at the same time. The larger error for 49.49 At% incorporation was found with both programs, and is caused by the increasing complexity of the spectra. As discussed by MacCoss et al.,[27] the width of the isotope distribution is broadest near 50 At% and narrowest at 0 and 100 At%, so that precision is higher in samples with more compact isotope distributions.

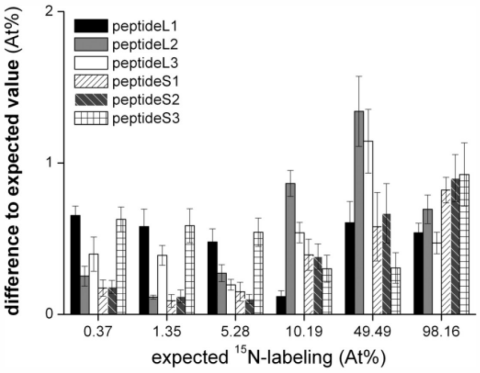

Figure 4. Absolute differences between calculated (MoLE) and expected ^{15}N-incorporation of RuBisCO from leaf extracts of plants grown at different concentrations of partial permanent ^{15}N-labeling. Mean ± SE (n = 5) of three peptides of RuBisCO LSU(L1−3) and SSU (S1−3) (for peptides see Table 1).

To further assess the quality of our measurements, we calculated the differences between two injections for five biological replicates using MoLE (Figure S4, Supporting Information). For low levels of labeling (<10.19 At%), these technical differences were less than 0.4 At%. At higher levels, the differences reached 1.0 At% (Figure S4). Overall, our data demonstrates, that ^{15}N-incorporation of the proteins can be reliably determined by MoLE.

Comparison of LC−MS^{E} and IRMS

The classical method to study nitrogen partitioning in plants is isotope ratio mass spectrometry (IRMS). IRMS measurements have a high precision (SD ≤ 0.3 % for ^{15}N) and instruments are usually optimized to natural abundance variations of isotopes.[53,54] We compared the ^{15}N-incorporation of RuBisCO LSU and SSU estimated by the program MoLE from LC−MS^{E} data and the ^{15}N-incorporation of total soluble protein (TSP) measured by elemental-analyzer continuous-flow IRMS (EA-CF-IRMS) with the expected values (Figure 5). Both approaches showed a clear linear relationship (R^2 = 0.9998) to the expected values, confirming the precision of our estimations.

However, for our research question, the analysis of ^{15}N-incorporation of proteins by LC−MS^{E} measurements has clear advantages. Samples for IRMS measurement with a labeling higher than 5 At% had to be diluted with unlabeled BSA to remain in the linear dynamic range of the IRMS instrument, and samples with a labeling of more than 10 At% could not be measured accurately by IRMS (data not shown). The difference from the expected value (Figure S5, Supporting Information) and the variability between the technical replicates (Figure S6) increased with the labeling, due to the difficulty of exact weighing in the submicrogram range during dilutions with BSA. Furthermore, IRMS requires tedious sample preparation in order to measure the ^{15}N-incorporation of individual proteins, and requires at least 300 ng of protein for accurate measurements which can only be extracted from multiple samples. Summarizing, in comparison with the IRMS, our approach demonstrated a high precision for ^{15}N-incorporation determination. IRMS is suitable for the determination of ^{15}N-incorporation in tissues or total soluble protein with a higher precision at lower ^{15}N-abundance, while our method also allows calculating ^{15}N-

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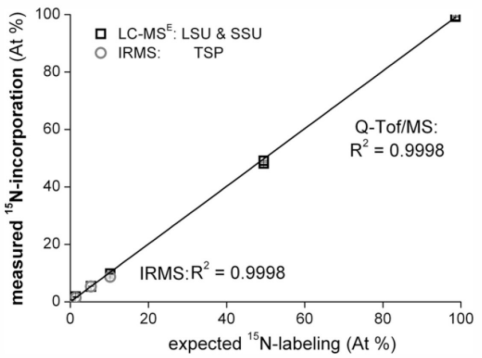

Figure 5. Correlation of calculated and expected ^{15}N-incorporation levels at different concentrations of partial permanent ^{15}N-labeling determined by two different methods. ^{15}N-incorporation of RuBisCO LSU and SSU (mean ± SE, n = 5) was calculated by MoLE algorithm from data measured by LC−MS^E and of total soluble protein it was determined by isotope ratio mass spectrometry (IRMS). Linear curve fit between expected and calculated ^{15}N-incorporation was 0.9998 for both methods.

incorporations of a large number of single proteins in complex mixtures also with high ^{15}N-abundances.

Quantitation of Isotopically Labeled Proteins after *in vivo* Pulse Labeling of Whole Plants Grown in Soil

Many previously published MS-based methods for quantitation of plant proteins were performed with plant cell suspension cultures,[16,17] hydroponic culture[51] or solid culture media.[21] However, plants grown in hydroponic culture have a limited value in studying ecological interactions;[55] and for the study of changes in protein pool sizes in plant-herbivore interactions a soil-based method is required. SILIP is the only MS proteomics method available for soil-grown plants,[14] but it requires a set of fully labeled and unlabeled plants which does not allow tracking of ^{15}N-flux and quantitation of proteins with unknown amounts of labeling. Therefore, we adapted the experimental design of van Dam and Baldwin,[4] who pulse-labeled plants grown in soil to study nitrogen acquisition after leaf removal and methyl jasmonate treatment.

Nicotiana attenuata plants grown in soil received a $K^{15}NO_3$ pulse of 5.1 mg nitrogen and were harvested at the indicated time-points after treatment (see also Materials and Methods) leading to different unknown levels of ^{15}N-incorporation. Three plants per time point received an unlabeled KNO_3 pulse to generate peptide lists with PLGS. On the basis of these peptide lists, we identified the most intense peptides of the proteins of interest (Table S3, Supporting Information) and determined the ^{15}N-incorporation of our three example proteins with MoLE algorithm.

A kinetic analysis revealed that the increase in labeling in the three analyzed proteins over time was exponential, and the best fit for describing the increase was obtained with a three parametric Gompertz function with a coefficient of correlation higher than 0.93 (Figure 6a). This relationship was also described for the increase in labeling of bacteria (*Pseudomonas putida*)[13] and for plant growth.[56] The maximal labeling (asymptote) was slightly different between the three proteins used in this study, but not statistically significant (data not shown). The slope for RCA2 was higher than for RuBisCO LSU and SSU (Figure 6a). The slope can be considered as measure for ^{15}N- incorporation

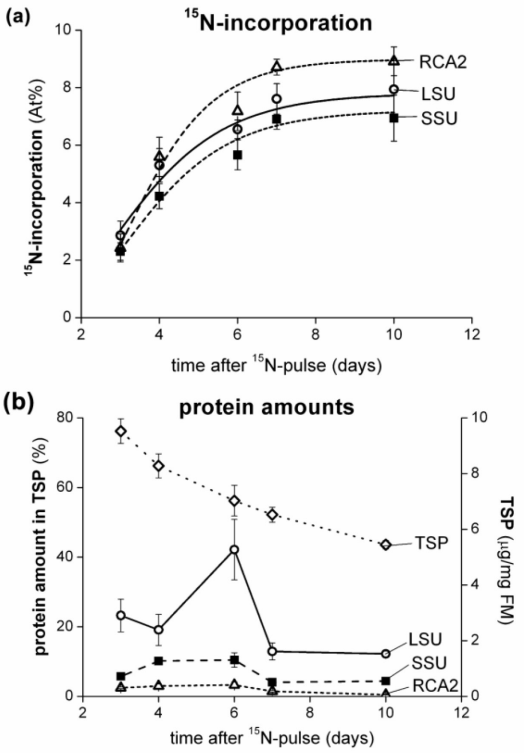

Figure 6. (a) ^{15}N-Incorporation and (b) protein amount in TSP of RuBisCO LSU and SSU and RCA2 over time from rosette leaves of soil-grown *N. attenuata* plants after *in vivo* labeling with $K^{15}NO_3$. The oldest sink leaves at the time-point of labeling were harvested at indicated time points (n = 5). Total soluble protein (TSP) extracts were measured by LC−MS^E. ^{15}N-Incorporation was determined with MoLE (R^2: RCA2 = 0.9650, LSU = 0.9262, SSU = 0.9695). The regression analysis was performed with Origin 8G. For ^{15}N-incorporation calculation and quantitation the most intense peptides (Table 1) were used. Mean ± SE are shown.

(metabolic turnover)[13] and gives a first hint that the turnover of RCA2 is faster than for RuBisCO. This result might be explained by the relationship between the proteins: RuBisCO can be activated very rapidly by RCA,[35] so that a faster fine-tuning of the amount of the activator is probably more important than the regulation of the RuBisCO amounts.

On the basis of the individual labeling of each peptide, we calculated the theoretical intensities of each peak of a spectrum of the peptides used for quantitation (Table S3, Supporting Information) and used them to estimate the TICs of the peptides. The average signal response calculated from the alpha trimmed means of the set of TICs of the peptides was used to quantify the three example proteins based on eq 1.2−1.4.

As expected, RCA2 was with values ranging from 0.45 to 3.28% of TSP less abundant than LSU (12.22 to 42.17% of TSP) and SSU (4.05 to 10.45% of TSP) (ANOVA, $F_{2,69}$ = 111.69, p < 0.001) which corresponds to ratios of RCA/RuBisCO between 0.03 and 0.1. This result is consistent with the findings for *N. tabacum* showing ratios of RCA/RuBisCO between 0.03 and 1.5.[48,57] Considering a limited amount of resources in plants, it is

sensible that the activator is available in lower amounts than the enzyme to be activated.

The time-course of the amounts of the three proteins in TSP showed different patterns (ANOVA, $F_{2,69} = 4.40$, $p < 0.05$), and did not correlate with the TSP per mg fresh mass which decreased with leaf age. The amount of RCA2 and RuBisCO SSU slightly decreased over time, while RuBisCO LSU showed a distinct increase 6 days after application of the ^{15}N-pulse (Figure 6b). In rice leaves RuBisCO contents have been shown to be dependent on the developmental stage of the leaf and to follow a log-normal curve,[58] thus, the increase in RuBisCO LSU might reflect the highest amount of RuBisCO at the full expansion of the leaf before senescence. This hypothesis is supported by Suzuki et al.,[59] who found maximal RuBisCO levels just before a rapid decline at full leaf expansion of *Eucalyptus globulus* seedlings. As climate and light conditions can only be partly regulated in the glasshouse and the increase in RuBisCO preceded a hot, sunny day, RuBisCO LSU amounts could have also been influenced by environmental conditions.[60] The amount of RuBisCO SSU did not show the transient increase supporting the findings in expanded tobacco leaves for which an SSU-independent control of LSU translation has been proposed,[61] we also cannot rule out that we missed an increase of RuBisCO SSU due to sampling times and the faster degradation of RuBisCO SSU compared to LSU.[62]

Overall our data confirm that enzyme pools and ^{15}N-incorporation levels can be reliably quantified after pulse-labeling of soil-grown plants enabling us to study further proteins relevant for plant-herbivore interactions. In contrast to other previously published MS-based methods for quantitation[14,16,17,21,51] the total number of samples is cut into half because the approach does not require the mix of an unlabeled and labeled sample. It only requires a single set of samples and three unlabeled samples per time point for peptide identification.

Quantitation of LOX2 Protein after Repeated Simulated Herbivory

As proof of principle of our approach and to demonstrate its applicability for ecological studies we analyzed the dynamics of LOX2 protein, an important enzyme for antiherbivore defense responses, after repeated herbivore treatment. During their lifetime, plants are often repeatedly challenged by herbivores, and the same herbivore usually feeds repeatedly on the same leaf or on different leaves of the same plant. It is known that an initial herbivore attack alters the plants ability to resist subsequent attacks.[39,63] It was hypothesized that a first attack leads to the accumulation of inactive forms of signaling proteins which are rapidly activated upon a subsequent attack.[39] However, little is known about the enzyme pools available in the plant after repeated herbivore feeding. In the past, identification and quantitation of the different LOX isoforms was mainly based on gene transcript data.[37,64] LOX proteins, in particular the JA-related LOX isoform (see Figure 7a), were primarily detected and quantified by antibodies,[65−67] but the use of antibodies only allows a limited throughput of samples and specificity has to be carefully evaluated. Additionally, in some studies, LOX activity was determined,[65,66] but this approach does not allow a distinction between different LOX isoforms.

Here we show for the first time the absolute quantitation of LOX2 protein in TSP. We treated wild tobacco leaves with standardized mechanical wounding and oral secretions from *Manduca sexta*, to simulate herbivory, and quantified LOX2 protein at different time-points after treatment. We elicited the plants every 24 h three times in a row. In addition to wild type plants we used plants impaired in JA-defense signaling (ir*LOX3*) in our analysis because gene expression of LOX2 in ir*LOX3* lines

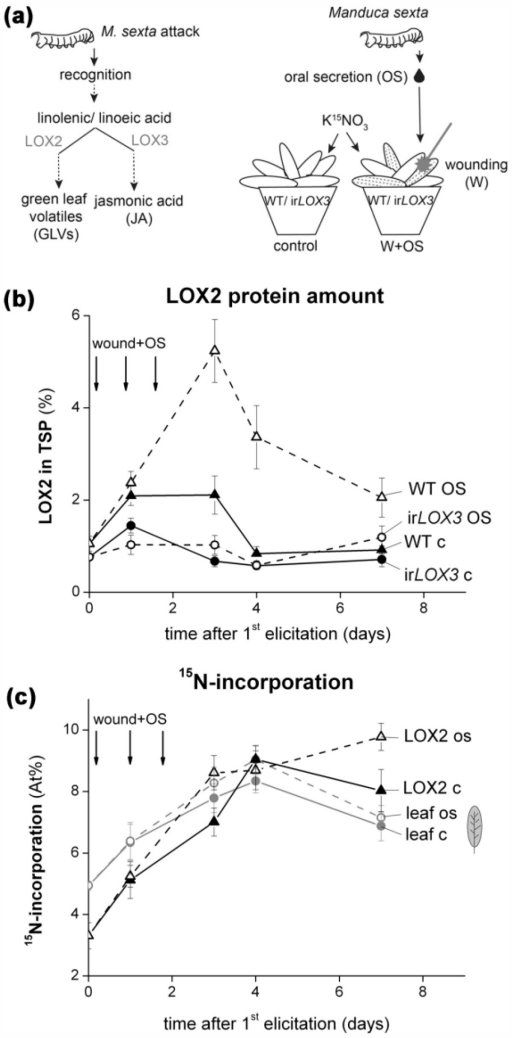

Figure 7. Role of lipoxygenase 2 (LOX2) after repeated herbivory. (a) Current simplified model of the role of LOX2 and a second LOX isoform, LOX3, after herbivory, and scheme of the experimental approach: Plants were pulse labeled with $K^{15}NO_3$ 7 days after transfer to 1 L pots and 3 days before wounding. The same three rosette leaves of ir*LOX3* and WT plants were wounded (W) with a pattern wheel and treated with 10 μL 1:5 diluted *Manduca sexta* oral secretion (OS) (W +OS) 3 days in a row. Untreated plants were taken as control. (b) LOX2 protein amounts in total soluble protein (TSP) (mean ± SE, $n = 5$) were measured by LC−MS^E and calculated based on the most intense peptides (Table S3, Supporting Information) as described for Figure 1. (c) ^{15}N-Incorporation of LOX2 protein in leaves of WT plants determined with MoLE and total ^{15}N-incorporation of the same leaf measured by IRMS. Arrows indicate days of treatment. Oldest sink leaves at time point of labeling were harvested at indicated time points.

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is reduced by 90% compared to WT plants after elicitation.[38] Thus, ir*LOX3* plants seemed to be the perfect tool for the validation of our quantitation approach.

LOX2 protein could be clearly identified on average with 17 peptides and a sequence coverage rate of 25.5%, while LOX3 protein could not be detected, neither in untreated nor in treated samples. We assume that the LOX3 protein is less abundant than LOX2. Peptides used for quantitation of LOX2 are shown in Table S3 (Supporting Information). All peptide sequences were carefully checked against the NCBI database to confirm their identity. LOX2 protein accounts for about 0.83 −2.1% of TSP in untreated WT plants (Figure 7b), while ir*LOX3* plants had in average significantly lower levels (59.3%) than WT (ANOVA, $F_{1,93} = 42.98$, $p < 0.001$, Figure 7b). The results clearly show that untreated plants already contain a substantial amount of the enzyme, and confirm the hypothesis that pre-existing enzyme pools are responsible and sufficient for the initial, rapid burst of GLV production after elicitation (20 min[38]).

LOX2 protein expression transiently increased 4 days after the first treatment (Figure 7b). This is first evidence, that plants are primed for the next attack by increasing basal enzyme levels. The increase only occurred after several treatments, consistent with the idea of Stork at al.[39] that after repeated herbivore feeding baseline levels of defense increase in discrete steps depending on the pattern of attack.

On the basis of our results we do not know if LOX2 protein accumulates in an active or inactive form. For AtLOX2, the NaLOX3 homologue in *Arabidopsis*, it was shown that untreated plants had an additional 26 amino acid transcript peptide compared to wound and salivary-treated plants.[68] In our data set we could not find an additional peptide sequence. Nevertheless, NaLOX2 might have to be activated by Ca^{2+} or by changing its phosphorylation status as assumed for AtLOX2 activity.[68,69]

In addition to the total protein amounts, we were interested in the ^{15}N-incorporation over time. We determined the ^{15}N-incorporation in the whole leaf by IRMS and in LOX2 protein by MoLE algorithm (Figure 7c). The values are in a similar range, but there was a time-shift. The overall uptake of ^{15}N into the leaf is faster than its incorporation into proteins. Furthermore, ^{15}N is more rapidly incorporated into LOX2 after attack than in control plants, indicating that the newly acquired nitrogen is partly channeled into LOX2 production. In ir*LOX3* plants the time-course of ^{15}N-incorporation in leaves and LOX2 protein was similar to WT plants, but the increase in LOX2 protein 7 days after simulated herbivory compared to control plants was more pronounced than in WT (Figure S7, Supporting Information).

■ CONCLUSION

These experiments demonstrate that our LC−MS^E-based approach allows a reliable quantitation of the leaf proteome with unknown ^{15}N incorporation levels based on the use of a single unlabeled internal standard. In comparison to other methods, ours is less expensive and generally applicable, because only one internal standard is used and moreover the internal standard BSA can be purchased in high quality and quantity at a low price, thus an experiment-specific synthesis, for example, of standard peptides, is not necessary. Sample preparation is easy and fast, and a high sample through-put is possible. Further automation to search for individual heavy peptides in spectra after bioinformatic identification of their natural forms is desirable, including spectral processing and output to MoLE.

We now have a tool at hand that enables us to compare in-depth the effects of herbivory on nitrogen distribution and protein pool sizes, broadening our picture of how silencing of specific genes relates to metabolism and defense.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Tables 1−3 and Supplemental Figures 1−7. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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SUPPLEMENTAL MATERIAL

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Table S1: ^{15}N-incorporation of the selected peptides of RuBisCO large (L1-3) and small subunit (S1-3) (Table 1) with different permanent ^{15}N-labeling of plants (value ± standard error (SE)); ^{15}N-incorporation was calculated A) with MoLE and B) with the excel sheet from (Taubert et al [19]); L1-3 and S1-3 are numbers of peptides (Table 1).

A

expected ^{15}N labeling [At%]	^{15}N-incorporation ± SE [At%] calculated by MoLE					
	0.37	**1.35**	**5.28**	**10.19**	**49.49**	**98.61**
L1	1.01 ± 0.06	1.93 ± 0.12	5.76 ± 0.09	10.11 ± 0.06	48.88 ± 0.14	99.15 ± 0.06
L2	0.61 ± 0.07	1.46 ± 0.01	5.01 ± 0.06	9.33 ± 0.09	48.15 ± 0.23	99.30 ± 0.09
L3	0.76 ± 0.11	1.74 ± 0.06	5.33 ± 0.10	9.65 ± 0.07	48.35 ± 0.21	99.08 ± 0.07
S1	0.53 ± 0.05	1.42 ± 0.05	5.16 ± 0.08	9.80 ± 0.10	49.03 ± 0.29	99.43 ± 0.08
S2	0.54 ± 0.04	1.47 ± 0.05	5.18 ± 0.03	9.81 ± 0.08	48.91 ± 0.25	98.96 ± 0.44
S3	0.99 ± 0.08	1.94 ± 0.11	5.82 ± 0.09	9.89 ± 0.09	49.39 ± 0.18	99.53 ± 0.21

B

expected ^{15}N labeling [At%]	^{15}N-incorporation ± SE [At%] calculated according to Taubert et al.					
	0.37	**1.35**	**5.28**	**10.19**	**49.49**	**98.61**
L1	0.87 ± 0.06	1.83 ± 0.09	5.47 ± 0.06	10.14 ± 0.04	48.89 ± 0.17	99.12 ± 0.07
L2	0.58 ± 0.06	1.41 ± 0.02	4.86 ± 0.21	9.30 ± 0.09	48.27 ± 0.21	99.07 ± 0.07
L3	0.46 ± 0.06	1.39 ± 0.02	4.91 ± 0.21	9.66 ± 0.09	47.74 ± 0.21	99.06 ± 0.10
S1	0.30 ± 0.03	1.33 ± 0.02	5.21 ± 0.08	9.63 ± 0.07	48.85 ± 0.22	98.95 ± 0.11
S2	0.48 ± 0.07	1.35 ± 0.03	5.17 ± 0.02	9.90 ± 0.09	48.58 ± 0.33	98.92 ± 0.14
S3	1.06 ± 0.06	2.00 ± 0.09	5.49 ± 0.05	10.18 ± 0.06	49.41 ± 0.23	99.00 ± 0.11

Table S2: Peptides of phosphorylase b from rabbit muscle and of BSA (B) used for calculation of the absolute protein quantitation on the column. The three most intense peptides were used. The table includes the average mass and retention time (Rt) calculated from 45-fold analysis. Carbamido methylated methionine residue is denoted as C*.

No.	Calc.$[MH]^+$	Exp. $[MH]^+$	Δ ppm*	Rt [min]	Rt RSD [%]	Sequence	Sumformula
P1	1853.9644	1853.9734	4.9	50.62	0.5	LLSYVDDEAFIRDVAK	$C_{84}H_{132}N_{20}O_{27}$
P2	1886.9031	1886.9120	4.7	45.46	0.6	GYNAQEYYDRIPELR	$C_{84}H_{123}N_{23}O_{27}$
P3	1678.8646	1678.8759	6.7	46.50	0.6	IGEEYISDLDQLRK	$C_{73}H_{119}N_{19}O_{26}$
B4	1163.6306	1163.6334	2.4	45.96	0.6	LVNELTEFAK	$C_{53}H_{86}N_{12}O_{17}$

B5	1419.6937	1419.7019	5.8	47.64	1.1	SLHTLFGDELC*K	$C_{62}H_{198}N_{16}O_{20}S_1$
B6	1639.9378	1639.9448	4.3	42.62	0.7	KVPQVSTPTLVEVSR	$C_{72}H_{126}N_{20}O_{23}$

Table S3: Peptides of ribulose-1,5-bisphosphate-carboxylase/oxygenases (RuBisCO) LSU (L) and small (S) SSU and RCA2 (R) and lipoxygenase 2 (LO) from *Nicotiana attenuata* and of BSA (B) used for absolute protein quantitation and for calculation of the ^{15}N-incorporation of soil grown plants pulse labeled with $K^{15}NO_3$. The three most intense peptides were taken, except from RuBisCO LSU where the 3th, 6th and 8th most intense peptides were used. The table includes the average mass and retention time (Rt) calculated from 13-fold analysis. Carbamido methylated methionine residue is denoted as C*.

No.	**Calc.$[MH]^+$**	**Exp. $[MH]^+$**	**Δ ppm***	**Rt [min]**	**Rt RSD [%]**	**Sequence**	**Sumformula**
L4	1261.7150	1261.7141	0.7	51.88	0.0	DITLGFVDLLR	$C_{58}H_{96}N_{14}O_{17}$
L5	1261.6285	1261.6303	1.4	42.02	0.4	FLFC*AEALYK	$C_{61}H_{88}N_{12}O_{15}S_1$
L6	1546.7358	1546.7371	0.8	41.65	0.0	WSPELAAAC*EVWK	$C_{71}H_{103}N_{17}O_{20}S_1$
S3	933.5152	933.5159	0.8	34.13	0.0	IIGFDNVR	$C_{42}H_{68}N_{12}O_{12}$
S4	1802.8781	1802.8811	1.7	40.22	0.0	QVQC*ISFIAYKPEGY	$C_{83}H_{123}N_{19}O_{24}S_1$
S5	893.4978	893.4964	1.6	32.85	0.1	EVEYLLK	$C_{42}H_{68}N_8O_{13}$
B7	1305.7161	1305.7172	0.8	31.66	0.0	HLVDEPQNLIK	$C_{58}H_{96}N_{16}O_{18}$
B8	1163.6306	1163.6325	1.6	36.52	0.0	LVNELTEFAK	$C_{53}H_{86}N_{12}O_{17}$
B9	1479.7954	1479.7962	0.5	40.81	0.0	LGEYGFQNALIVR	$C_{68}H_{106}N_{18}O_{19}$
LO1	1142.6051	1142.6052	0.0	35.66	0.0	EALPEDLISR	$C_{49}H_{84}N_{13}O_{18}$
LO2	1572.8631	1572.8665	2.2	47.85	0.0	DVLLFETPELLQR	$C_{72}H_{117}N_{17}O_{22}$
LO3	1629.8370	1629.8397	1.7	34.99	0.0	LDPEIYGPPESAITK	$C_{74}H_{116}N_{16}O_{25}$
R4	1882.9697	1882.9745	2.5	49.83	0.0	IVDTFPGQSIDFFGALR	$C_{88}H_{131}N_{21}O_{25}$
R5	1706.7980	1706.8001	1.2	35.78	0.0	GLVQDFSDDQQDIAR	$C_{71}H_{111}N_{21}O_{28}$
R6	1332.6794	1332.6796	0.2	34.05	0.0	WVSGTGIEAIGDK	$C_{59}H_{93}N_{15}O_{20}$

***Δ ppm=10^6 *(M_{tn}-M_{exp})*M_{tn}^{-1}**

LEGENDS:

Figure S1: Fertilization scheme of a) permanent labeling experiment and b) pulse labeling experiment. a) 12 days after germination plants were transferred to 50 mL single pots with different concentrations of $Ca(^{15}NO_3)_2$ (see Material and Methods). 10 days later they were put into 1 L single pots with the same concentrations of ^{15}N in the form of $K^{15}NO_3$. Ten days later plants were harvested. b) 7 days after transfer to 1 L pots, plants were pulse labeled with $K_{15}NO^3$. Three days later was the first time-point of harvest.

Figure S2: LC-MS^E production spectra of selected peptides (Table 1) a-c) for LSU; d-f) for SSU; g-i) for RCA2; j-l) for BSA2; pe = precursor error

Figure S3: Absolute difference between calculated (excel sheet Taubert et al. [19]) and expected ^{15}N-incorporation at different concentrations of partial permanent ^{15}N-labeling. Mean ± SE (n=5) of three peptides of RuBisCO LSU (L1-3) and SSU (S1-3) (for peptides see Table 1).

Figure 4: Absolute differences of the ^{15}N-incorporation of RuBisCO peptides between technical replicates determined with MoLE from leaf extracts of plants grown at different concentrations of partial permanent ^{15}N-labeling. Mean ± SE (n=5) of the difference between two technical replicates is shown (for peptides see Table 1).

Figure S5: Absolute difference between measured and expected ^{15}N-incorporation of total soluble protein determined by IRMS from leaf extracts of plants grown at different concentrations of partial permanent ^{15}N-labeling. The proteins with an expected ^{15}N-incorporation higher than 5 % were mixed with BSA before analysis to dilute the labeling to about 1 At% ^{15}N-labeling. Mean ± SE (n=5) of the differences is shown.

Figure S6: Absolute differences between technical replicates of the ^{15}N-incorporation in TSP measured with IRMS from leaf extracts of plants grown at different concentrations of partial permanent ^{15}N-labeling. Mean ± SE (n=5) of the difference between two technical replicates is shown. Samples with a labeling higher than 5 % were mixed with BSA before analysis to dilute the labeling to about 1 At% ^{15}N-labeling.

Figure S7: ^{15}N-incorporation of LOX2 protein in leaves of ir*LOX3* plants determined with MoLE and total ^{15}N-incorporation of the same leaf measured by IRMS.Arrows indicate days of treatment. Oldest sink leaves at time point of labeling were harvested at indicated time points. For further details see Fig. 7.

Figure S1

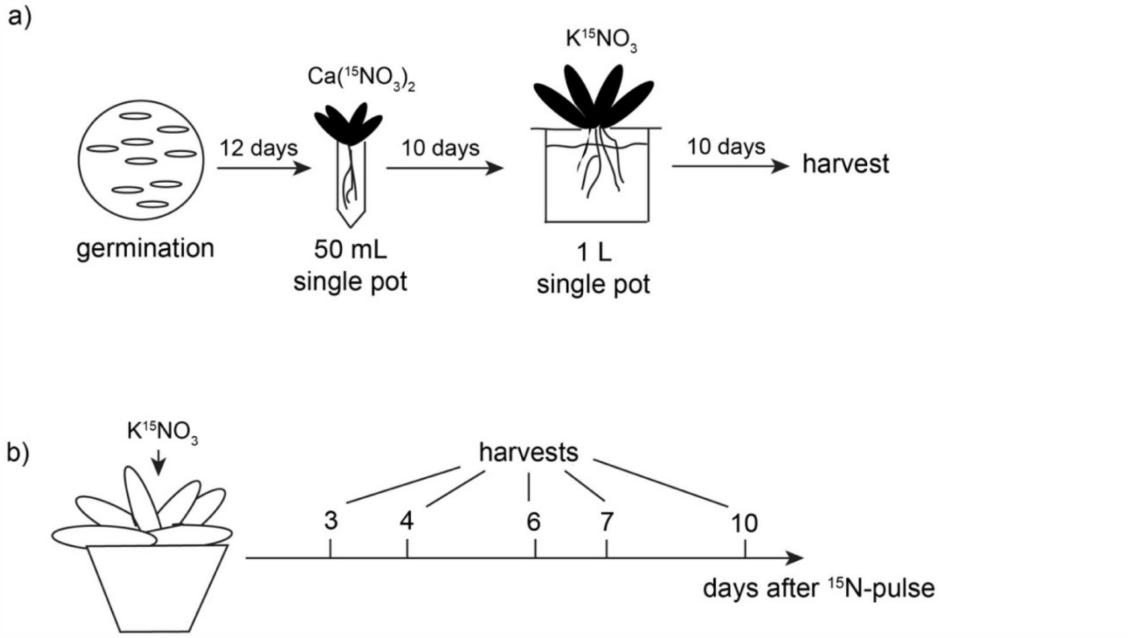

a)
$K^{15}NO_3$
$Ca(^{15}NO_3)_2$
12 days
10 days
10 days
harvest
germination
50 mL
single pot
1 L
single pot
b)
$K^{15}NO_3$
harvests
3
4
6
7
10
days after ^{15}N-pulse

Figure S2

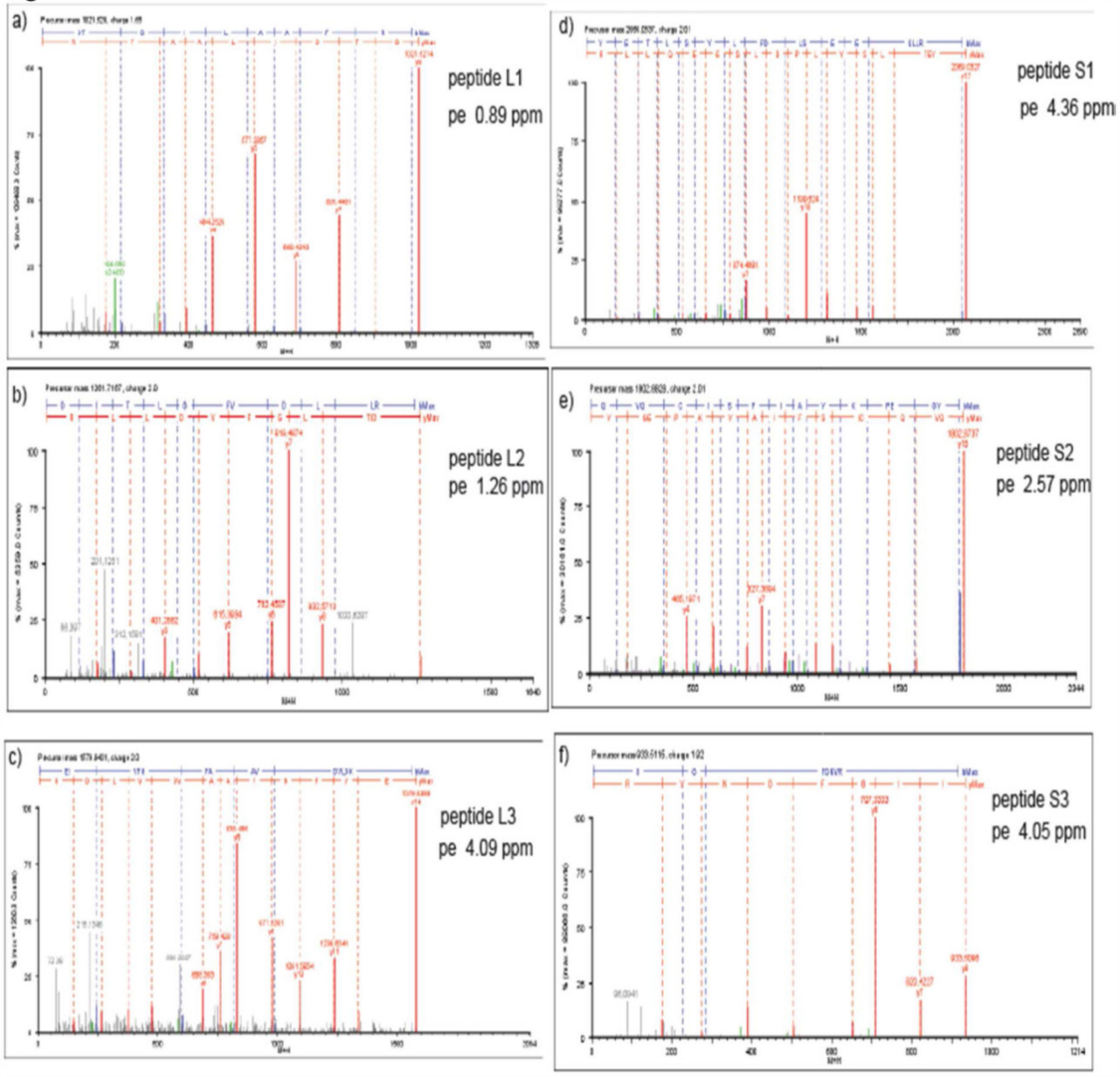

a)
peptide L1
pe 0.89 ppm
d)
peptide S1
pe 4.36 ppm
b)
peptide L2
pe 1.26 ppm
e)
peptide S2
pe 2.57 ppm
c)
peptide L3
pe 4.09 ppm
f)
peptide S3
pe 4.05 ppm

Figure S2 continued

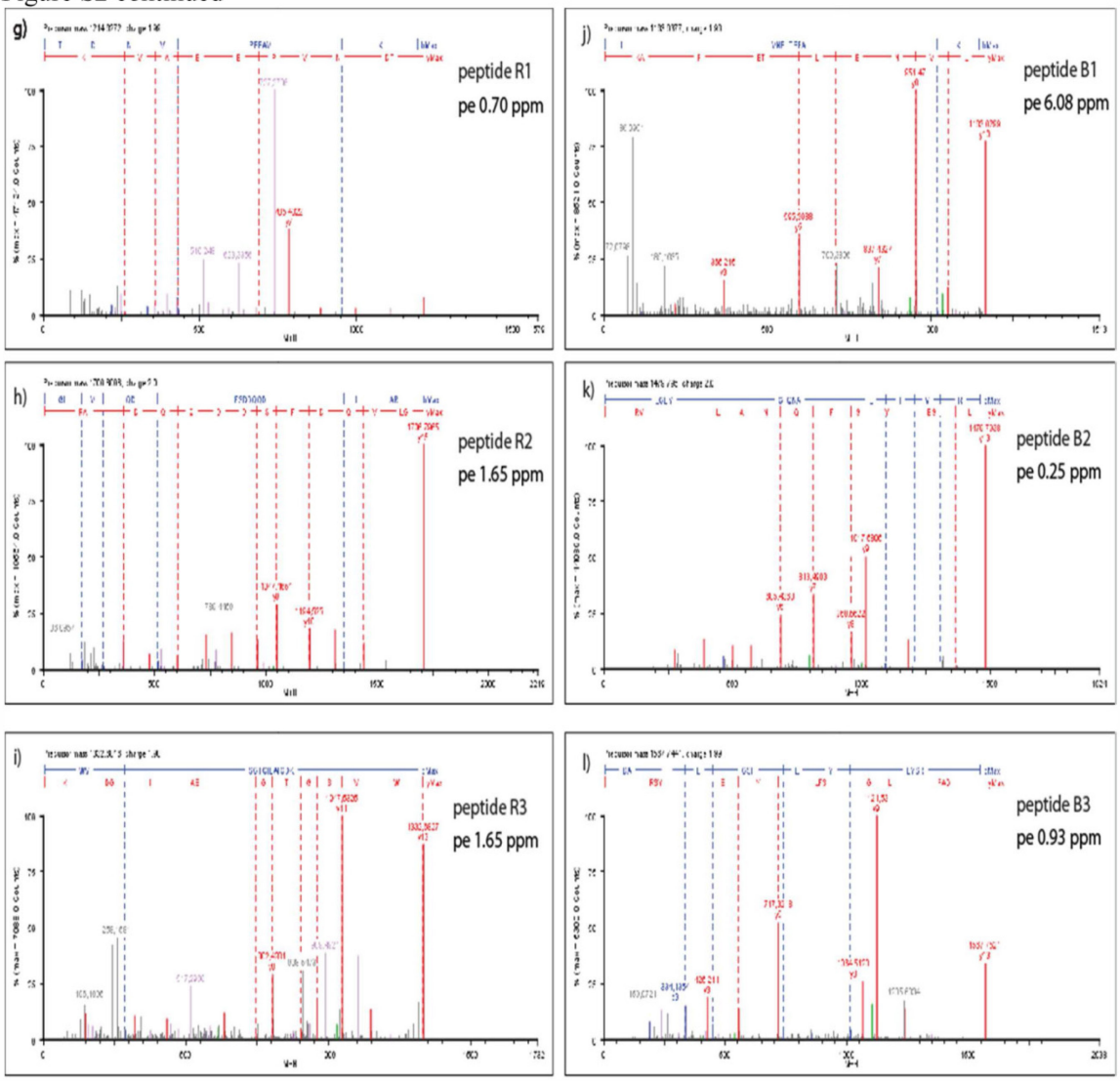
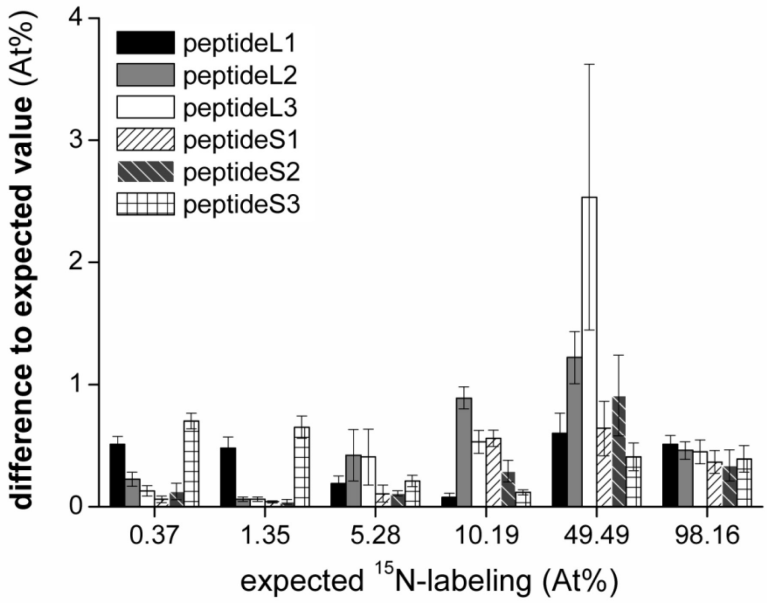


Figure S3


peptideL1
peptideL2
peptideL3
peptideS1
peptideS2
peptideS3
difference to expected value (At%)
0
1
2
3
4
0.37
1.35
5.28
10.19
49.49
98.16
expected 15N-labeling (At%)

7

Figure S4

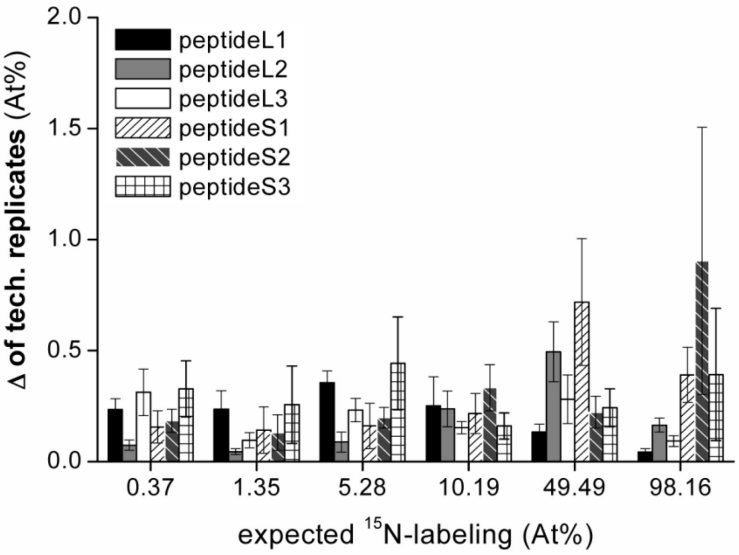
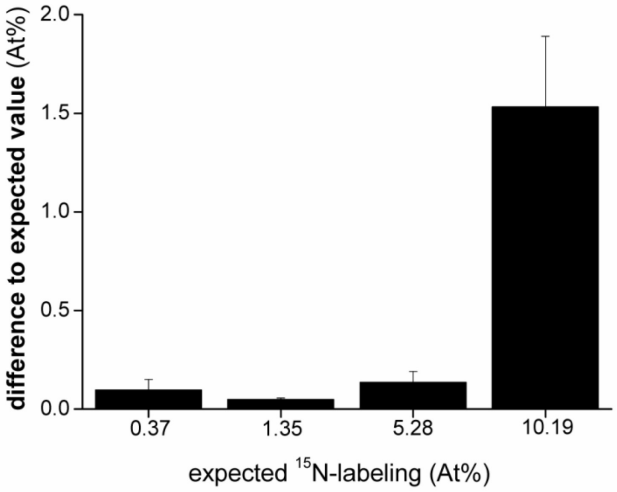

peptideL1
peptideL2
peptideL3
peptideS1
peptideS2
peptideS3
Δ of tech. replicates (At%)
2.0
1.5
1.0
0.5
0.0
0.37
1.35
5.28
10.19
49.49
98.16
expected 15N-labeling (At%)

Figure S5


difference to expected value (At%)
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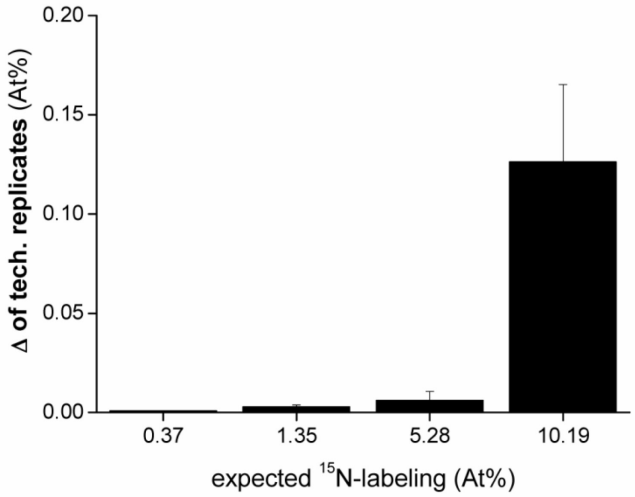
0.5
0.0
0.37
1.35
5.28
10.19
expected 15N-labeling (At%)

Figure S6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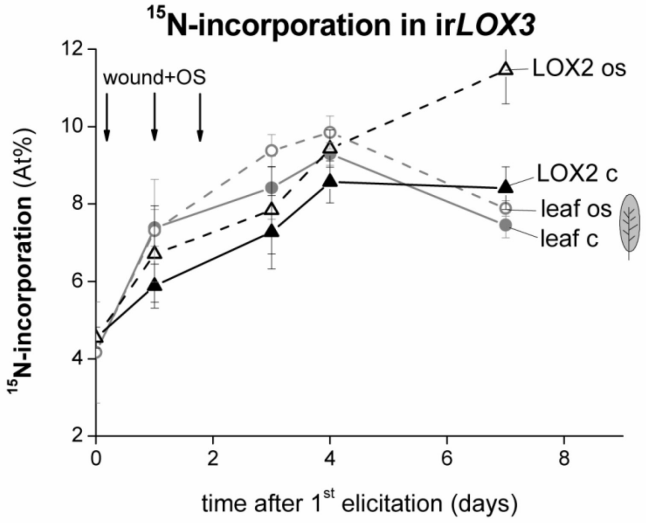
0.15
0.10
0.05
0.00
Δ of tech. replicates (At%)
0.37
1.35
5.28
10.19
expected 15N-labeling (At%)

Figure S7


15N-incorporation in irLOX3
wound+OS
LOX2 os
LOX2 c
leaf os
leaf c
15N-incorporation (At%)
time after 1st elicitation (days)
12
10
8
6
4
2
0
2
4
6
8

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Quantification of growth–defense trade-offs in a common currency: nitrogen required for phenolamide biosynthesis is not derived from ribulose-1,5-bisphosphate carboxylase/oxygenase turnover

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SUMMARY

Induced defenses are thought to be economical: growth and fitness-limiting resources are only invested into defenses when needed. To date, this putative growth–defense trade-off has not been quantified in a common currency at the level of individual compounds. Here, a quantification method for ^{15}N–labeled proteins enabled a direct comparison of nitrogen (N) allocation to proteins, specifically, ribulose-1,5-bisposphate carboxylase/oxygenase (RuBisCO), as proxy for growth, with that to small N-containing defense metabolites (nicotine and phenolamides), as proxies for defense after herbivory. After repeated simulated herbivory, total N decreased in the shoots of wild-type (WT) *Nicotiana attenuata* plants, but not in two transgenic lines impaired in jasmonate defense signaling (ir*LOX3*) and phenolamide biosynthesis (ir*MYB8*). N was reallocated among different compounds within elicited rosette leaves: in the WT, a strong decrease in total soluble protein (TSP) and RuBisCO was accompanied by an increase in defense metabolites, ir*LOX3* showed a similar, albeit attenuated, pattern, whereas ir*MYB8* rosette leaves were the least responsive to elicitation, with overall higher levels of RuBisCO. Induced defenses were higher in the older compared with the younger rosette leaves, supporting the hypothesis that tissue developmental stage influences defense investments. We propose that MYB8, probably by regulating the production of phenolamides, indirectly mediates protein pool sizes after herbivory. Although the decrease in absolute N invested in TSP and RuBisCO elicited by simulated herbivory was much larger than the N-requirements of nicotine and phenolamide biosynthesis, ^{15}N flux studies revealed that N for phenolamide synthesis originates from recently assimilated N, rather than from RuBisCO turnover.

Keywords: *Nicotiana attenuata*, caffeoyl-putrescine, dicaffeoyl-spermidine, nicotine, ribulose-1,5-bisphosphate carboxylase/oxygenase, total soluble protein, R2R3-MYB transcription factor, *Manduca sexta*.

INTRODUCTION

Plants have evolved two general direct strategies against herbivory: constitutive and inducible defenses. The biosynthesis of these defenses requires fitness-limiting resources that could otherwise be invested into growth and reproduction. Hence, induced plant defenses are thought to be a cost-saving strategy compared with constitutive defenses, as they are only produced when needed, e.g. after herbivory (Karban and Baldwin, 1997), and this cost-saving model plays a central role in most theoretical treatments of induced defenses (for a review of plant defense hypotheses, see Stamp, 2003). Several studies have quantified the costs of induction by measuring photosynthesis rates, plant bio-

mass, size and/or yield associated with an increase in defense metabolites (Bazzaz *et al.*, 1987; Karban and Baldwin, 1997; Zangerl *et al.*, 2002). Although measurements of the impact of anti-herbivore defenses on plant yield are important for understanding their ultimate fitness costs, measurements of plant biomass do not discriminate among the relative investments into compounds that function in growth, storage and defense processes in the tissues analyzed (Chapin *et al.*, 1990). Therefore, the investment into growth is preferably estimated by measuring components of biomass that directly promote the acquisition of resources for growth, such as photosynthetic proteins (Chapin *et al.*, 1990). Additionally, the costs of defense should be measured in the currency of a fitness-limiting resource (Mole, 1994; Baldwin *et al.*, 1998). Nitrogen (N) is often such a fitness-limiting resource, determining the growth and reproduction of plants, and of the herbivores that eat them. N availability also influences N allocation to defense metabolites (Baldwin *et al.*, 1998; Lou and Baldwin, 2004; Simon *et al.*, 2010). Thus, it is an ideal currency to use for the study of growth–defense trade-offs in plant–herbivore interactions.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the most abundant foliar protein in plants, and is essential for the dark reaction of photosynthesis. RuBisCO constitutes 30–50% of the total soluble protein (TSP) in C3 plants (Ellis, 1979; Makino *et al.*, 1984; Imai *et al.*,2008), and may function as a potential N storage protein (Millard, 1988); consequently, it represents a major N sink in plants. Its large and small subunits (LSUs and SSUs, respectively) are synthesized from separate precursor pools that have different metabolic origins (Allen *et al.*, 2012). Although the concentration and activity of RuBisCO are not the only factors controlling growth (Stitt and Schulze, 1994), changes in RuBisCO expression influence growth and lead to complex changes in N metabolism (Stitt and Schulze, 1994; Stitt and Krapp, 1999; Matt *et al.*, 2002), making this enzyme a reasonable proxy for growth parameters.

Nicotiana attenuata is a wild tobacco native to the Great Basin Desert in south-western USA that synchronizes its germination from long-lived seed banks in response to exposure to cues from pyrolized vegetation (Preston and Baldwin, 1999). By timing its germination with the immediate post-fire environment, *N. attenuata* takes advantage of the abundant, yet ephemeral, pools of inorganic N in burned soil (Lynds and Baldwin, 1998), but is subject to high intraspecific competition for this fitness-limiting resource because of its mass-germination behavior. Furthermore, because it is a pioneer species, *N. attenuata* is attacked by a diverse herbivore community, including the specialist tobacco hornworm (*Manduca sexta*). Herbivore attack elicits the jasmonic acid (JA) signaling cascade (Kessler *et al.*, 2004), which activates JA-responsive transcription factors that lead to the biosynthesis of a plethora of induced small metabolites (Figure 1a; Woldemariam *et al.*, 2011), such as the N-intensive alkaloid nicotine, and a variety of phenolamides, which decrease herbivore performance (Baldwin, 1999; Steppuhn *et al.*, 2004; Kaur *et al.*, 2010; Onkokesung *et al.*, 2010). The biosynthesis of nicotine and phenolamides requires the same amino acid precursors (ornithine and arginine for putrescine and spermidine biosynthesis; Kaur *et al.*, 2010; Steppuhn *et al.*, 2004; Takano *et al.*, 2012), but nicotine is produced only in the roots (Hibi *et al.*, 1994), whereas phenolamides are synthesized in the attacked leaf (Kaur *et al.*, 2010).

Nicotine is present constitutively in undamaged *N. attenuata* tissues, and foliar concentrations increase substantially after herbivory (McCloud and Baldwin, 1997; Baldwin, 1999). The two major phenolamides found in *N. attenuata* are the N-acylated polyamines caffeoylputrescine (CP) and dicaffeoyl-spermidine (DCS), the biosynthesis of which is regulated by the transcription factor NaMYB8 (hereafter MYB8; Figure 1a). Both CP and DCS accumulate constitutively in reproductive tissues, and are strongly induced in leaves by simulated herbivory (Kaur *et al.*, 2010). Herbivory also causes large-scale changes in *N. attenuata*'s transcriptome and proteome, decreasing the levels of photosynthetic genes and proteins, including RuBisCO (Halitschke *et al.*, 2003; Voelckel and Baldwin, 2004b; Giri *et al.*, 2006). Because of the important constraints imposed by N availability upon both its growth and defense, as well as the wealth of understanding of its anti-herbivore defenses and the availability of isogenic transgenic lines impaired in individual classes of defenses, *N. attenuata* is an ideal model in which to study growth–defense trade-offs in a common N currency.

The induction of defense responses in wild tobacco can be simulated in a standardized and synchronized way by wounding leaves and applying the oral secretions (OS) of *M. sexta* larvae to the wounds (W + OS, Figure 1). The major elicitors in *M. sexta* OS are fatty-acid amino acid conjugates (FACs), which are recognized by the plant, triggering defense responses (Schittko *et al.*, 2001; Halitschke *et al.*, 2003; Giri *et al.*, 2006). The FAC composition of OS, and the resulting gene expression and metabolite induction in the plant, differ between specialist and generalist folivores (Voelckel and Baldwin, 2004a; Diezel *et al.*, 2009; Steinbrenner *et al.*, 2011).

Here, we quantified the N investments into different plant parts and among different N pools within a tissue to compare the investments into growth and defense in the same currency after repeated simulated herbivory by W + OS elicitation from a specialist herbivore. Repeated simulated herbivory, in contrast to single W + OS elicitation, more closely mimics natural herbivore feeding, which varies in duration and timing (Van Dam *et al.*, 2001; Skibbe *et al.*, 2008; Stork *et al.*,2009). A stable isotope labeling technique was used to track N flux among different pools

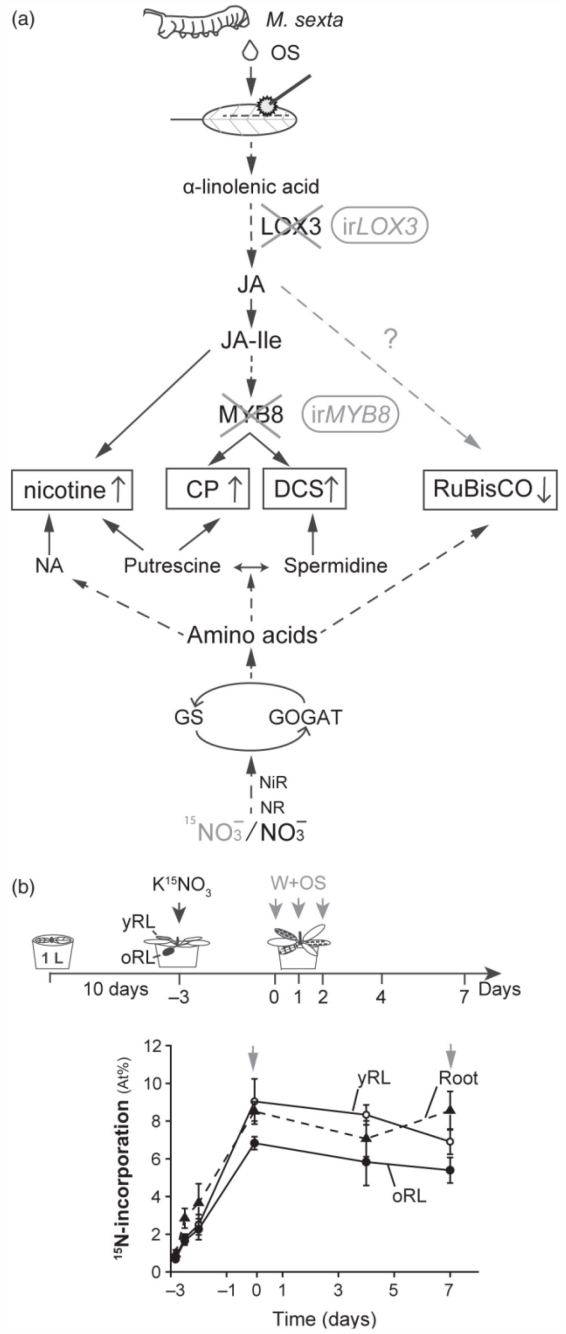

Figure 1. Overview of experimental strategy used to study growth–defense trade-offs in *Nicotiana attenuata* in a common nitrogen (N) currency.
(a) The biosynthesis of nicotine, caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS) is induced after simulated herbivory in the wild type (WT) by wounding (W) with a pattern wheel and by the application of oral secretions (OS) of *Manduca sexta*, but is impaired in the transgenic plants silenced in the expression of *lipoxygenase 3* (*LOX3*) or *MYB8* by RNAi with inverted-repeat (ir) constructs. The concentration of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) decreases in the WT after W + OS, but the effects of jasmonic acid (JA) on N investment into RuBisCO are unclear. Amino acids serve as precursors for putrescine, spermidine and nicotinic acid (NA), which provide N for the synthesis of these metabolites. Amino acids are derived from nitrate (NO_3^-) reduction, followed by assimilation catalyzed by glutamine synthetase (GS) and glutamate synthase (GOGAT), and are also used as precursors for RuBisCO synthesis. JA-Ile, JA-isoleucine; NiR, nitrite reductase; NR, nitrate reductase.
(b) Incorporation of ^{15}N into roots, and younger (yRL) and older rosette leaves (oRL) following pulse labeling with $K^{15}NO_3$ 27 days after germination was determined by isotope-ratio mass spectrometry (IRMS; $n = 5$). Grey arrows indicate the time points of elicitation in the experiments that followed. During this time frame ^{15}N-incorporation was stable. At%, atomic percentage.

of individual compounds in locally elicited and systemic leaves and seeds. We applied ^{15}N-labeled nitrate to the soil because nitrate is the most common form of N taken up by *N. attenuata* in nature, after the rapid biological nitrification of the ammonium generated by pyrolysis (Figure 1; Lynds and Baldwin, 1998).

The N flux into the three major N-intensive small metabolites of *N. attenuata* (nicotine, CP and DCS) was used as a proxy for defense investment that could be directly compared with the N investment into proteins, and in particular the abundant photosynthetic protein, RuBisCO, as a proxy for growth-related investment. These different molecule classes could not be measured in the past with comparable precision and accuracy because of a lack of suitable methods, especially for proteins. Here, we used a high-throughput LC-MS^E method for the absolute quantitation of proteins and the incorporation of ^{15}N into peptides (Ullmann-Zeunert *et al.*, 2012), which allows for the quantification of single large proteins with the same accuracy as for the small defense metabolites quantified by UPLC/UV/ToF-MS.

To further disentangle the effects of induced defenses on N allocation after herbivory, we compared two previously described transgenic lines, one deficient in JA signaling, ir*LOX*3 (Allmann *et al.*, 2010), and one deficient in the biosynthesis of phenolamides, ir*MYB8* (Kaur *et al.*, 2010), with wild-type (WT) plants (Figure 1a). This design allows for a direct comparison of N flux into specific classes of defense compounds with that into growth-related proteins measured in the same N currency, and an evaluation of the hypothesis that RuBisCO is used as an N-storage compound for defense responses.

RESULTS AND DISCUSSION

Anti-herbivore defense elicitation alters the nitrogen content of the shoot

Herbivory is known to change resource allocation within plants (Bazzaz *et al.*, 1987; Frost and Hunter, 2008; Gomez *et al.*, 2010). To estimate the impact of the biosynthesis of N-containing defense metabolites on N accumulation in *N. attenuata*, we compared the shoot N contents (% dry mass) of the two transgenic lines impaired in defense

responses with WT plants after repeated simulated herbivory with W + OS. The isotope ratio mass spectrometry (IRMS) measurements revealed that repeated elicitation reduced the N content of WT shoots (i.e. the total of N per dry mass of shoots; Welch's two-sample *t*-test, d.f. = 7.24, $P = 0.032$), but not of the transgenic lines (Figure 2), whereas the N pool sizes were slightly reduced after elicitation for all three genotypes (Figure S1b). The changes in the N pool sizes of elicited ir*LOX3* and ir*MYB8* plants were the result of a reduction in shoot dry mass (Figure S1), whereas the elicited WT showed both reduced shoot dry mass and reduced shoot N content, suggesting a possible N reallocation within the plant caused by the biosynthesis of N-containing defense metabolites.

Plants can allocate N to roots to protect this resource from folivores to reduce the nutritional value of the attacked tissues, which, together with increased defenses, can slow herbivore growth and increase their exposure to natural enemies (Trumble *et al.*, 1993). Previous studies with *Solanum lycopersicum* (tomato) demonstrated that N allocation in the form of amino acids from the shoot to the roots was rapidly induced by methyl-jasmonate (MeJA; Gomez *et al.*, 2010) and *M. sexta* feeding (Steinbrenner *et al.*, 2011; Gomez *et al.*, 2012). In *N. attenuata*, OS elicitation has been shown to cause a rapid allocation of carbon from the shoot to the roots, which can later be used for regrowth and reflowering (Schwachtje *et al.*, 2006). The reduced N concentration of WT shoots in our experiment suggests that this species can also allocate N from the shoot to the roots after herbivory. This inference is consistent with the observation that the N contents of WT roots increased after elicitation, as measured in a separate experiment, although the increase was not quite significant (Welch's two-sample *t*-test, d.f. = 4.71, $P = 0.054$; inset Figure 2). Alternatively, the increased N content of roots may have resulted from increased N assimilation, but previous ^{15}N labeling experiments in this species have found no evidence for changes in N assimilation rate after herbivory (Baldwin and Ohnmeiss, 1994; Lynds and Baldwin, 1998). Therefore, we conclude that the induced biosynthesis of N-containing metabolites after OS elicitation alters whole-plant N partitioning.

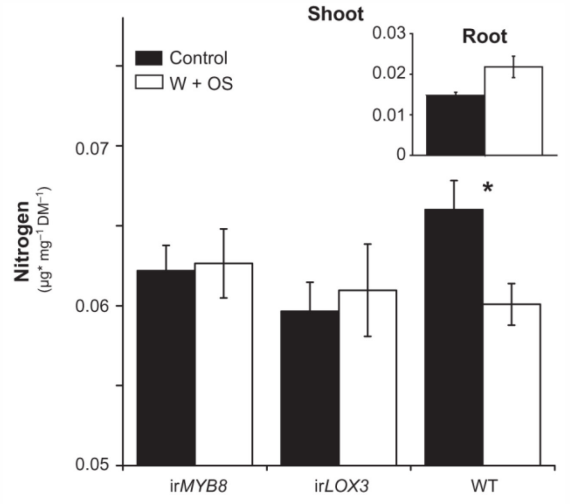

Figure 2. Total nitrogen (N) content in wild-type (WT) shoots decreases after simulated herbivory.
The N content of shoots of ir*LOX3*, ir*MYB8* and WT ($n = 5$) was determined by IRMS 4 days after the first W + OS elicitation. Unelicited plants were controls. Asterisks represent significant differences between treatments ($*P \leq 0.05$; $n = 5$). Inset: N content of WT roots ($n = 5$) was determined in a separate experiment at the same time point. DM, dry mass.

Changes in absolute pool sizes depend on developmental stage

To analyze the influence of anti-herbivore defense induction, especially phenolamide biosynthesis, on within-shoot N allocation, we determined the absolute N pools of different leaf types (hereafter, total N pools) and N allocation to seeds by IRMS. Expressing resource allocation as concentrations reveals proportional allocations within an organ; however, total pools allow for comparisons among organs, as they are a function of both organ size and concentration (Chapin *et al.*, 1990). We analyzed elicited older (oRL) and younger (yRL) rosette leaves to explore the influence of leaf development on N reallocation after elicitation, and the first (unelicited) stem leaf (S1) to examine systemic effects.

Overall, there was no clear effect of genotype or elicitation on the leaf total N pools. Total N pools varied among genotypes only in the S1 leaf (ANOVA, $F1_{,27} = 4.86$, $P = 0.036$), whereas OS elicitation only reduced the total N pool of ir*LOX3* (two-sample *t*-test, d.f. = 8, $P = 0.006$) and WT (Welch's two-sample *t*-test, d.f. = 8, $P = 0.021$) in the yRL (ANOVA, $F1_{,28} = 7.40$, $P = 0.011$). The N pool size in oRL was unaffected by genotype and elicitation (Figure 3). As N pool size correlates with biomass at the whole-plant scale (Baldwin and Hamilton, 2000), we evaluated whether the observed changes in total N pools of single leaves could be explained by changes in growth. Although the leaf size of yRL was reduced after elicitation (ANOVA, $F1_{,24} = 12.33$, $P = 0.002$; Figure S2a), it did not correlate with total N pools (ANCOVA, $P = 0.187$). Similarly, the change in total N pools of S1 leaves was not correlated with changes in leaf size (ANCOVA, $P = 0.406$).

It is possible that changes in the total N pool of a leaf reflect changes in a major pool within the leaf, such as proteins. Although TSP pool size dramatically decreased in the yRL after elicitation, it did not correlate with the total N pool size in this tissue (ANCOVA, $P = 0.122$; Figure 3). Thus, we conclude that although both pools are reduced by elicitation, the total N pool of the rosette leaves does not reflect the changes in TSP pool size or leaf size. This result

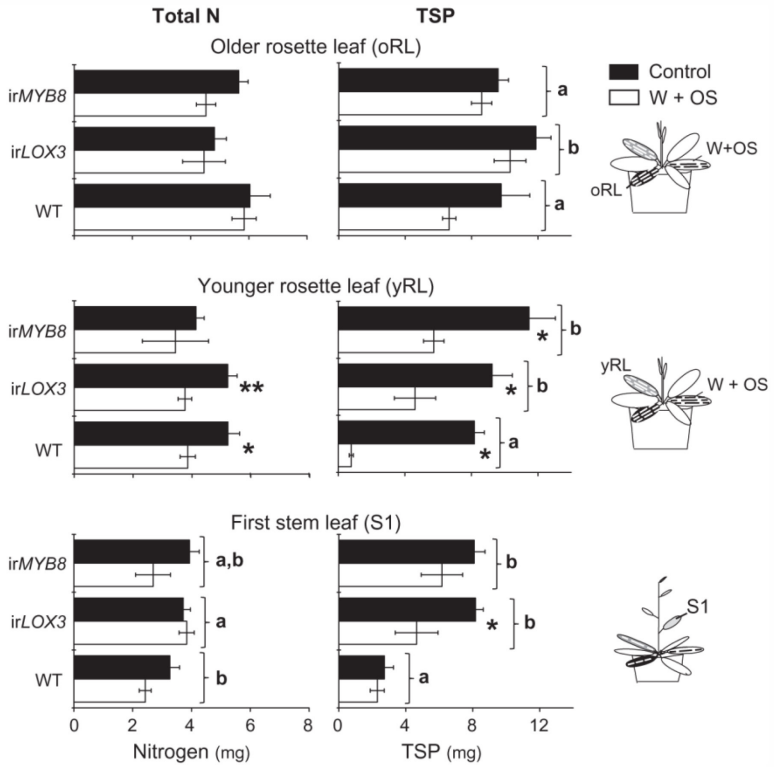

Figure 3. Silencing of *LOX3* and *MYB8* alters the distribution of nitrogen (N) between and within leaves. The N pools and total soluble protein (TSP) of leaves (oRL, older rosette leaf; yRL, younger rosette leaf; S1, first stem leaf) of ir*LOX3*, ir*MYB8* and WT, calculated based on leaf mass. The N content was determined by IRMS and the TSP was measured by the Bradford assay. Plants were elicited as described for Figure 2. yRL and oRL were harvested 4 days after the first W + OS elicitation, and when S1 leaves underwent the source–sink transition. Asterisks indicate differences among treatments (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Letters represent significant differences found using the minimum adequate model ($n = 5$). For abbreviations, see Figure 1.

is consistent with the hypothesis that total leaf N content and TSP (RuBisCO content) are controlled by different mechanisms, as has been shown for *Oryza sativa* (rice; Ishimaru *et al.*, 2001; Makino *et al.*, 2000).

The TSP pools differed between the lines in all three leaf types (ANOVA; oRL, $F_{1,27} = 8.70$, $P = 0.007$; yRL, $F_{1,27} = 12.95$, $P = 0.001$; S1, $F_{1,27} = 44.77$, $P = 3.5*10^{-07}$; Figure 3). The TSP pools of ir*LOX3* and ir*MYB8* in the yRL were reduced by about 50% after OS elicitation, whereas WT TSP pools were reduced by 91%. Both transgenic lines had constitutively larger TSP pools in the S1 leaf than the WT, and although S1 TSP decreased after elicitation in ir*LOX3* plants, TSP pools of both transgenic lines were still 2.5–3.0 times larger than those of WT after elicitation (Figure 3), suggesting that the biosynthesis of N-containing metabolites affects protein pool sizes, and that inducible defenses also have constitutive costs.

The recently developed method for the absolute quantification of single proteins allowed us to quantitatively compare the investment in defense metabolites with that in growth-related compounds, specifically, the photosynthetic protein RuBisCO, with similar accuracy (Ullmann-Zeunert *et al.*, 2012). Being the most abundant soluble protein in plants, the total level of RuBisCO (sum of LSU and SSU) reflected the TSP pattern in the different leaves, independent of genotypes (ANCOVA; oRL, $P < 0.0001$; yRL, $P < 0.0001$; S1, $P = 0.42$; Figures 3 and S3a). Overall, the data revealed a decrease in pool sizes of both RuBisCO subunits after OS elicitation (Figure S3a), which coincided with an increase in N-containing defense metabolites (Figure S3b), but the effects differed among lines, and for most traits measured ir*LOX3* showed an intermediate phenotype between WT and ir*MYB8*. The two transgenic lines with either reduced (ir*LOX3*) or undetectable levels of CP and DCS (ir*MYB8*; Figure S3b) showed a smaller decrease of RuBisCO LSU and SSU than the WT in the elicited yRL (47–59% in ir*MYB8*/ir*LOX3* compared with 92–95% in WT; Figure S3a). RuBisCO LSU and SSU levels were unaltered after elicitation in the systemic S1 leaf of ir*MYB8*, but strongly declined in WT and ir*LOX3*. The nicotine pool sizes showed similar induction patterns for all lines, except in the yRL, where the OS-elicited nicotine levels were higher in the WT than in the transgenic lines. These data suggest that the growth–defense trade-offs at the leaf scale are probably influenced by the capacity to biosynthesize and accumulate phenolamides, and that this also affects growth investments in the systemic S1 leaf.

As all transgenic lines used in this study accumulated similar levels of nicotine, it is unclear whether the biosynthesis of this alkaloid might affect N allocation to proteins

(Figure S3b). To answer this question rigorously, experiments with transgenic lines completely with no flux of N into nicotine biosynthesis are needed. In the nicotine-silenced transgenic lines we have produced in our laboratory by silencing putrescine N-methyl transferase, nicotine biosynthesis is silenced, but the elicited flux of N into other alkaloids (anatabine) is not (Steppuhn *et al.*, 2004).

Similarly, the induction of proteinase inhibitors could have additional influence on N allocation; however, preliminary experiments with virus-induced empty vector and *MYB8*-silenced plants showed a similar trypsin proteinase activity in both plants after elicitation (H. Kaur, personal communication), indicating that the synthesis of proteinase inhibitors does not seem to play a key role in the reallocation of N from primary to secondary metabolism.

A comparison of the two locally elicited leaves revealed differences in their defense and growth pool sizes: whereas the oRL accumulated the largest defense metabolite pools, with only slight reductions in TSP and both RuBisCO subunits after elicitation, the yRL had the strongest reductions in protein pools, with less pronounced increases in N-containing defense metabolite levels than the oRL (Figures 3 and S3). The optimal defense theory predicts that the allocation of defense metabolites is directly proportional to the fitness value of different plant parts (McKey, 1974, 1979; Rhoades, 1979), and many studies have demonstrated that younger leaves of *N. attenuata*, presumed to have a higher fitness value than older leaves, contain higher defense metabolite levels (Zavala *et al.*, 2004a; Kaur *et al.*, 2010; Onkokesung *et al.*, 2012). These results appear to contradict our findings, because the oRL contained higher metabolite levels than the yRL; however, the previous studies compared concentrations of metabolites in elicited rosette leaves at different stages of plant development, whereas here we analyzed metabolite pool sizes of two elicited rosette leaves, of different maturity, harvested simultaneously from the same plant. As the plants were just beginning stalk elongation at the time of OS elicitation, both oRL and yRL are likely to be important tissues for later plant growth and reproduction. Thus the larger defense metabolite pools of the mature oRL – which was a source leaf at the time of the first elicitation – may result from its larger nutrient pools, which are probably important for regrowth capacity. Meanwhile, the smaller pools of TSP and RuBisCO in elicited yRL – which was in the transition stage from sink to source during the first W + OS treatment – may reflect a lower N allocation to proteins in developing leaves, which could enhance their defense status by reducing the food quality for herbivores. This is in agreement with the model from Orians *et al.* (2011), assuming that the mature source leaf allocates resources not only to defense and growth, but also to storage, thus making it relatively more valuable for the whole plant, and therefore better protected. Regardless of their ultimate explanations, these data demonstrate that growth–defense trade-offs are dependent on leaf development.

Many previous studies have demonstrated that inducible defenses are costly, often leading to a decrease in reproductive performance (Heil and Baldwin, 2002): e.g. growth–defense trade-offs at the leaf scale affect the N allocation to capsules in *Nicotiana sylvestris* (Ohnmeiss and Baldwin, 2000). However, here, neither the time of flowering and seed ripening, nor the number of mature capsules, the mass of the first mature seed capsule, nor the total N content of the first seed capsule were significantly different from controls after repeated simulated herbivory (Figure S4). This lack of observed fitness effects could result from species-specific differences or differences in the experimental design. In our experiment, OS elicitation may have been too early to affect seed set (the first capsules were harvested on average 18 days after the last elicitation), or the W + OS treatment was too weak to elicit changes in allocation to seeds, compared with the relatively stronger MeJA elicitation used in other experiments (Voelckel *et al.*, 2001). In nature, wild tobacco faces strong intraspecific competition because of its mass-germination behavior, and strong alterations in N allocation to reproductive units in glasshouse-cultivated tobacco were only found when MeJA-elicited plants competed with control plants for the same limited resources (Van Dam and Baldwin, 2001). Thus, the costs and benefits of N allocation for a plant after herbivore attack may only become obvious if neighboring plants competing for the same limited resources are present. Additional experiments with plants grown in competition and exposed to simulated and natural herbivory are necessary to further explore the impact of growth–defense trade-offs within the leaf on plant fitness.

MYB8 indirectly affects nitrogen investment into proteins

The pool sizes of proteins and defense metabolites of the two transgenic lines suggest an influence of N-containing metabolite biosynthesis on the observed growth–defense trade-offs, but did not allow for a direct comparison of the levels of N demanded for metabolite biosynthesis, and the decreased N partitioned into TSP and RuBisCO after herbivory. By calculating the N investment into growth and defense per mg of fresh tissue mass after elicitation, we were able to further explore the role of phenolamide biosynthesis on N reallocation. We combined this approach with ^{15}N pulse labeling to follow the investment of a defined N pool into both plant functions.

For all lines, and in locally treated leaves, elicitation decreased the N investment into rest TSP and RuBisCO per mg fresh mass, compared with controls. Particularly in yRLs, the decrease in N investment into TSP (rest TSP and RuBisCO) was much more pronounced in the WT (89%)

compared with 28% in ir*MYB8* and 47% in ir*LOX3* (Figure 4a). Ir*LOX3* plants, for all parameters measured here, showed similar but less pronounced N-allocation patterns after elicitation as WT. These patterns are consistent with the correlation analysis of all measured N pools (Figure 4a, heat maps). Correlating all genotype/treatment groups with each other revealed that OS-elicited WT plants did not correlate with the other genotype/treatment groups in all three leaf types. Only OS-elicited ir*LOX3* oRL and S1 leaves showed a weak correlation with WT-OS. In contrast, ir*MYB8*-OS did not correlate with any other genotype by treatment group.

Interestingly, the observed N-investment pattern is congruent with previous results on the patterns of *MYB8* transcript accumulation in *N. attenuata* as*LOX3* plants (which are comparable with ir*LOX3*; Allmann *et al.*, 2010; Halitschke *et al.*, 2004). After elicitation, as*LOX3* leaves have four times lower *MYB8* transcript levels, whereas ir*MYB8* have 10 times lower levels than WT leaves (Kaur *et al.*, 2010; Onkokesung *et al.*, 2012). Furthermore, MYB8

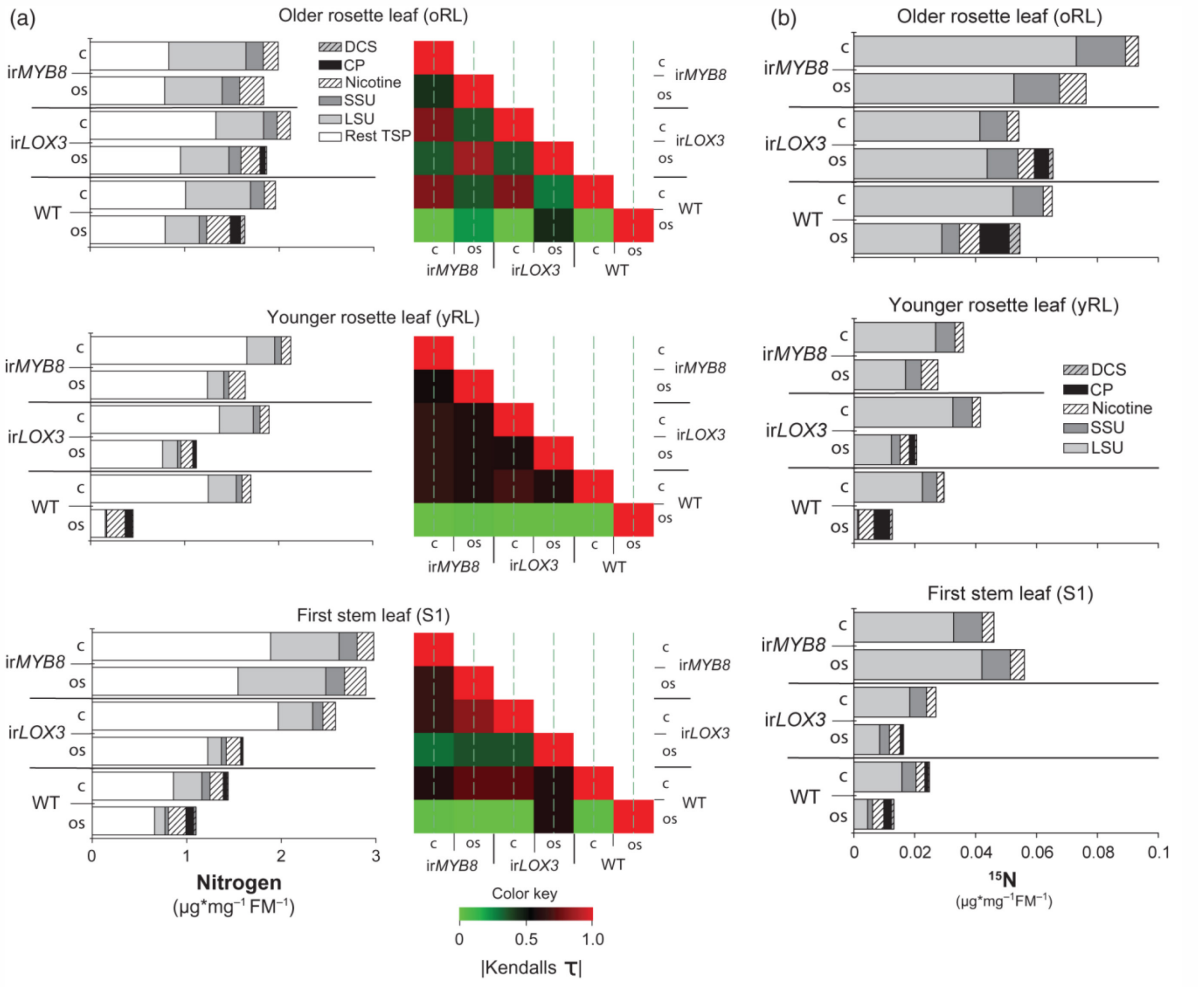

Figure 4. The increased nitrogen (N) investment in nicotine, caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS) is accompanied by a decreased N investment in protein.

(a) Investment of N in residual total soluble protein (TSP) [TSP − (SSU + LSU)], RuBisCO large (LSU) and small (SSU) subunits, nicotine, CP and DCS in older rosette leaves (oRL), younger rosette leaves (yRL) and first stem leaves (S1) was calculated by multiplying the proportion of N in each compound with the concentration of the compound for each leaf. The level of TSP was quantified by the Bradford assay, RuBisCO LSU and SSU were determined by LC-MS^E, and the defense metabolites were determined by UPLC-UV-ToF-MS. Plants were elicited as described in Figure 2 and leaves were harvested as described in Figure 3 (n = 5). FM, fresh mass; for other abbreviations, see Figure 1. Heat maps represent Kendall's τ coefficient for pairwise correlation of N investment in all of the above compounds among all genotype/elicitation groups.

(b) Investment of ^{15}N in RuBisCO LSU and SSU and defense metabolites was calculated as ^{15}N-incorporation multiplied by the N investment. Plants were pulse-labeled with $K^{15}NO_3$ 3 days before the first treatment. ^{15}N-incorporation was determined based on the MS spectra with the Excel spreadsheet ProSIPQuant (Taubert *et al.*, 2011).

functions downstream of JA signaling, and OS-elicited JA levels are not altered in ir*MYB8* plants (Kaur *et al.*, 2010), whereas they are significantly reduced in *LOX3*-silenced lines (to about one-third of that in the WT, but roughly six to seven times higher than in untreated controls; Allmann *et al.*, 2010). Thus, the observed phenotypes of the two transgenic lines are consistent with their respective MYB8 transcript levels, but not their JA levels; the *MYB8* expression after elicitation in the three lines used in this study is inversely proportional to the N investments into soluble proteins. Based on these results we conclude that the observed changes in N allocation after simulated herbivory only indirectly depend on JA signaling, and are probably caused by differences in *MYB8* expression or the MYB8-regulated synthesis of phenolamides. MYB8 could regulate defense induction by playing a role in N assimilation and allocation. In other plants and algae, members of the R2R3-MYB transcription factor family, to which NaMYB8 belongs, have been shown to be crucial for increases in the abundance of transcripts of N assimilation genes (Miyake *et al.*, 2003; Imamura *et al.*, 2009). To further elucidate the putative role of MYB8 in N reallocation, more detailed expression and enzyme activity studies targeting N metabolism at later time points after herbivory are necessary.

Based on our data we cannot differentiate whether MYB8 itself or the synthesis of phenolamides, in particular CP and DCS, mediate the changes in N investment into growth and defense. Silencing *MYB8* also silences genes further downstream of the transcription factor, and in addition to CP and DCS, the synthesis of at least 29 different coumaroyl-, caffeoyl- and feruloyl-containing metabolites (Onkokesung *et al.*, 2012). It is difficult to pinpoint the effects of single compounds in the complex biosynthetic network of a leaf, but applying phenolamides in different concentrations to control and elicited leaves of ir*MYB8* plants, and evaluating their effects on protein (RuBisCO) levels, or using plants silenced in genes affecting phenolamide biosynthesis downstream of MYB8, can help to evaluate if either MYB8 alone or MYB8 indirectly through phenolamide biosynthesis mediates the changes in N investment into proteins.

A comparison of the total N investment with the ^{15}N investment per mg fresh mass revealed a similar pattern, with increased ^{15}N in defense compounds and decreased ^{15}N in both RuBisCO subunits after elicitation. One major difference was that WT and ir*LOX3* plants allocated proportionally more ^{15}N than total N into CP and DCS, and less into nicotine, after elicitation, whereas the ^{15}N investment into the RuBisCO subunits was proportionally similar to the total N investment in both control and elicited leaves (Figure 4b; for a clearer comparison of N and ^{15}N investment, see Figure S5). Larger investments of recently assimilated ^{15}N into CP and DCS, compared with nicotine, makes ecological sense, because the OS used was from *M. sexta* larvae, a tobacco specialist, which is nicotine-tolerant but negatively affected by phenolamides (Kaur *et al.*, 2010).

A comparison of the decrease in total N investment into RuBisCO and TSP after OS elicitation with the N requirements of nicotine and phenolamide biosynthesis (Figures 4a and S6, showing a time-course analysis) suggested that RuBisCO metabolism could be a source of reallocated N to defense metabolite biosynthesis. Based on concentrations in the yRL, about 54% of N from RuBisCO or 13% of N from TSP could have been invested into phenolamides and nicotine (Figure S5). This comparison does not take into account the N requirements of biosynthetic enzymes or other N-containing inducible defense compounds, such as proteinase inhibitors (Zavala *et al.*, 2004b). Hence, the N demands for defense metabolite biosynthesis are likely to be underestimated; however, considering the dramatic decline in TSP it is likely that more N is released from the turnover of primary metabolism than N invested into defense metabolites.

Nitrogen invested into phenolamides does not originate from RuBisCO after herbivory

To further elucidate the N flux into defense metabolites and to investigate whether RuBisCO N is used as a source of N for CP and DCS biosynthesis after OS elicitation, the ^{15}N-incorporation (atomic percentage, At%) into N-containing metabolites and RuBisCO was determined in a time-course experiment (for details, see Figure 1b). This approach allows us to follow the N flux of a known quantity of ^{15}N, independently of within-leaf N pool sizes. The experiment was carried out with the yRL, because this leaf showed the greatest differences in N investment after elicitation (Figure 4a,b). It is important to note that during the experimental period, the ^{15}N-incorporation of the whole leaf was constant in all three lines, independent of elicitation (Figure S7), indicating that N is mainly redistributed within the leaves and that there is no increased net N influx into the leaf after elicitation.

As the ^{15}N-incorporation into RuBisCO LSUs and SSUs was similar, we only report on the incorporation into LSUs. Incorporation into RuBisCO increased at a constant rate until it reached a maximum of about 8 At% between 4 and 7 days after the first OS elicitation in all three lines, independent of elicitation (Figure 5). In contrast, ^{15}N was rapidly incorporated into CP and DCS in OS-elicited leaves until these compounds attained a maximum of about 10–12 At%, 4 days after the first elicitation in WT and ir*LOX3* plants. Had RuBisCO degradation provided the precursors for PA biosynthesis, it should have a similar or higher ^{15}N-incorporation as phenolamides, because the precursor pools will have similar or higher labeled isotope incorporation rates as their derived compounds. The large differences in ^{15}N-incorporation between CP and DCS and

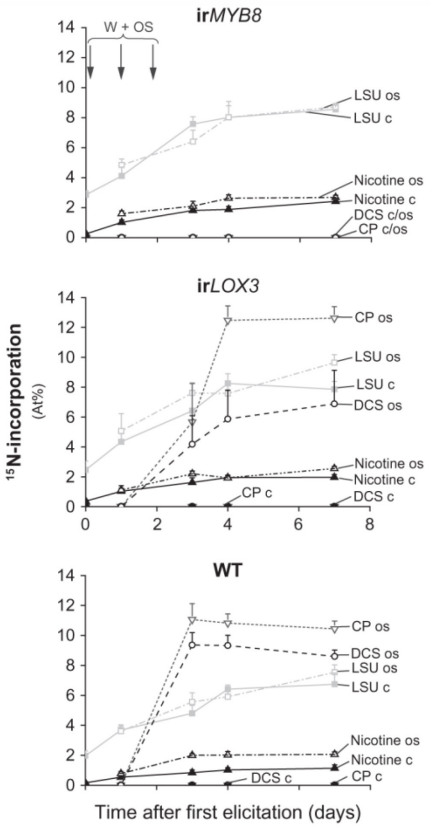

Figure 5. The dynamics of ^{15}N-incorporation into nicotine, caffeoyl-putrescine (CP), dicaffeoyl-spermidine (DCS) and RuBisCO large subunit (LSU) demonstrates that recently assimilated N, not N derived from LSU metabolism, is rapidly invested into CP and DCS biosynthesis after elicitation. Three days before the first W + OS treatment plants were pulse-labeled with $K^{15}NO_3$ (see Figure 1a). The yRL at the time of labeling was harvested at the time points indicated. ^{15}N-incorporation ($n = 5$) of RuBisCO LSU, nicotine, CP and DCS was determined as described for Figure 4. For abbreviations see Figure 1.

LSU make it unlikely that N derived from RuBisCO was used for CP and DCS biosynthesis. This result challenges the common conception that N released from products of primary metabolism (proteins) is a direct source for the production of defense metabolites (Herms and Mattson, 1992; Schwachtje *et al.*, 2006). In contrast, the data indicate that recently assimilated N is channeled into defense metabolite synthesis (Figure 4b). We hypothesize that N released from TSP turnover is mainly reinvested into other compounds, enabling the plant to react in different ways upon attack. Thus, plants may reduce the nutritive value of the tissue by reducing the level of TSP, and at the same time investing N not only in defense metabolites, but also in other N-containing compounds that are less digestible for the herbivore or more easily reallocated.

The incorporation of ^{15}N into nicotine only increased slightly after elicitation, and reached a maximum of around 2 At% in all three lines (Figure 5), although roots had a labeling of about 8 At%, similar to leaves (Figure S6). These findings differ from previous results showing the rapid incorporation of recently assimilated ^{15}N into nicotine after elicitation, but those results were obtained from plants that were starved of N for 24 h before application of the ^{15}N pulse, and ^{15}N was applied at the same time as MeJA to the roots (Baldwin *et al.*, 1994, 1998; Lynds and Baldwin, 1998). Elicitation of roots and shoots is known to differentially affect the accumulation of defense metabolites (van Dam and Oomen, 2008). Furthermore, MeJA is a stronger elicitor than OS elicitation (Voelckel *et al.*, 2001), and N-starved plants are known to transport N preferentially to the strongest sink (Ohtake *et al.*, 2001). These differences in experimental design probably led to different source–sink relationships within the plant, resulting in different patterns of ^{15}N investments.

Nicotine is a constitutively synthesized pool in the roots of *N. attenuata* that is transported to the shoot, but not metabolized, and contains 5–8% of the total N in the plant (Baldwin and Hamilton, 2000). It is possible that the newly synthesized nicotine might be diluted by the large pool of previously synthesized unlabeled nicotine, resulting in a low ^{15}N-incorporation. Alternatively, it may be derived from previously synthesized (and therefore unlabeled) precursors.

In summary, the ^{15}N-incoporation illustrates the flux of a defined ^{15}N pulse, independent of pool size, and indicates that N invested into CP and DCS is unlikely to be derived from RuBisCO, but is allocated directly to defense processes after assimilation instead of growth processes.

CONCLUSION

In this study, we quantified simulated herbivory-induced growth–defense trade-offs in a unified currency by measuring the investments of the limited resource of N into RuBisCO as proxy for growth and into small defense-related compounds (nicotine and phenolamides). In *N. attenuata*, OS elicitation reconfigures N allocation on multiple scales. Figure 6 summarizes the relative changes in the different N pool sizes after repeated simulated herbivory in the yRL. At the whole-plant scale, OS elicitation induced a weak N reallocation from the shoot to the root, thus presumably giving attacked plants a higher tolerance against herbivores by reducing the chances that valuable resources are removed by herbivores, and by increasing regrowth capacity after attack. At the within-leaf scale, changes between different N pools are much more dramatic. Taking the N level of RuBisCO after elicitation as a reference (x), RuBisCO-N declined 21x, and TSP declined from 73x to 9x, whereas N investment into defense metabolites increased, but to a far lesser extent (an increase from 5.5x to 11.5x for nicotine, and of 5x for CP and DCS).

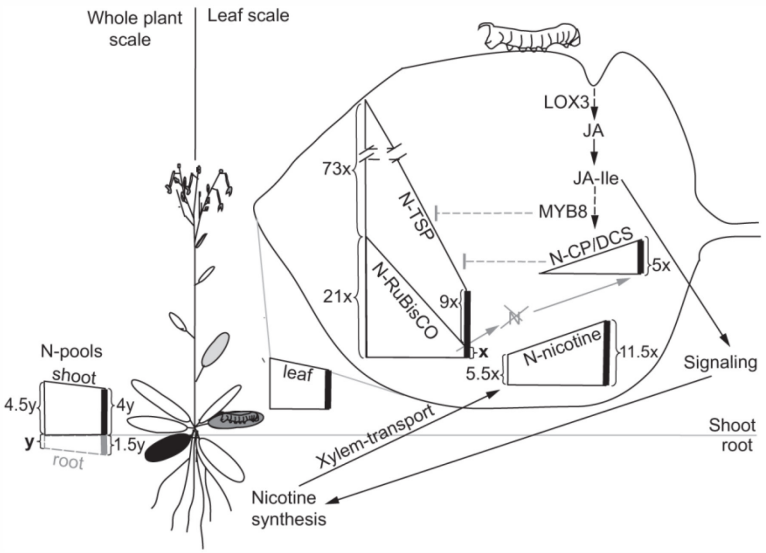

Figure 6. Herbivory-induced trade-offs of nitrogen (N) investment into growth and defense are mediated by MYB8. N investment in defense causes a reallocation of N from the shoot to the root. We suggest that the transcription factor MYB8, probably via the synthesis of phenolamides (caffeoyl-putrescine, CP; dicaffeoyl-spermidine, DCS), is involved in the reallocation of N within the local leaf. N invested in phenolamides, and in the root-synthesized alkaloid nicotine, increases after herbivory, whereas the N-investment in total soluble protein (TSP) and RuBisCO strongly decreases, but it is unlikely that N invested into phenolamides originates from RuBisCO metabolism. The height of the left and right side of the quadrangles represent relative changes in N pool sizes for each compound of C- and OS-elicited plants, respectively, using the N level of RuBisCO after elicitation as a reference (*x*). All N pools within the depicted leaf show the ratios of measured values per mg fresh mass. Shoot, root and whole-leaf N pools depicted outside the plant represent ratios of N determined per mg dry mass. For abbreviations, see Figure 1.

The transcription factor Na*MYB8*, possibly by regulating the production of metabolically dynamic phenolamides, CP and DCS, indirectly mediates the reconfiguration of N allocation after elicitation. The comparison of two elicited rosette leaves indicated that the extent of reconfiguration and the total concentration of defense metabolites produced depends on the developmental stage of the leaf, and on source–sink relationships.

Flux studies with ^{15}N strongly indicated that the N for PA biosynthesis comes from recently assimilated N rather than RuBisCO turnover. These results suggest that the drastic reallocation of resources and the shut-down of investments into growth within the leaf are not primarily driven by the direct costs for defense metabolite biosynthesis, but rather that N release from primary metabolism may enable the plants to react to attack in multiple ways. It remains to be elucidated if and how these allocation costs are translated into ecological costs. This question can only be answered if plants are grown in competition under different levels of herbivore attack.

Future experiments will seek to validate these results under more natural settings by comparing the results shown here for simulated herbivory, using OS of a specialist folivore, with damage by the natural herbivore community. An additional focus will be on tracing N investments into further metabolites and non-soluble proteins, and following the flux of N at the whole-plant level in more detail.

EXPERIMENTAL PROCEDURES

Plant germination and growth conditions

Seeds of the 31st generation of an inbred WT line of *N. attenuata* Torr. ex. Watts (Solanaceae) and two stably transformed lines, ir*MYB8* with reduced expression of the transcription factor Na*MYB8* (A-08-810, Kaur *et al.*, 2010), and ir*LOX3* silenced in lipoxygenase 3 (Na*LOX3*, A-03-562-2, Allmann *et al.*, 2010), were sterilized and germinated according to Kruegel *et al.* (2002) and cultivated in 1-L pots. For details on cultivation and fertilization, see the Appendix S1. The transgenic lines were homozygous, near-isogenic to the WT and representative of several independent transformation events.

Plant treatment

Pre-experiment to determine elicitation time points. Seven days after transfer to 1-L single pots, rosette-stage plants were pulse-labeled with 5.1 mg ^{15}N in 50 ml of a 0.694 g l^{-1} solution of $K^{15}NO_3$ (modified from Van Dam and Baldwin, 2001), and the oldest sink leaf (hereafter yRL) and the youngest source leaf (hereafter oRL; Pluskota *et al.*, 2007) were labeled for later sampling. The leaves and roots were harvested at 0, 4 and 12 h, and at 4, 7 and 10 days after the ^{15}N pulse. Roots were washed to remove excess soil and all samples were dried for 48 h at 60°C. Between 3 and 10 days after the pulse, the leaves and roots had a constant ^{15}N concentration (Figure 1b), indicating that an equilibrium had been reached. This time period was chosen for further experiments (Figure 1b, indicated by the grey arrows), as a stable ^{15}N-incorporation facilitates the analysis of proportional allocation to single compounds. The N pulse did not have any obvious effects on plant growth.

Pulse-labeling experiments. Three days after the ^{15}N pulse the oldest sink, youngest source and transition leaf at the time point of labeling were wounded with a pattern wheel and treated with *M. sexta* OS (10 μl per leaf per day, 1:5 diluted) on three consecutive days (Ullmann-Zeunert *et al.*, 2012). Unelicited plants were used as controls. For the whole-shoot N analysis, the aboveground biomass of control and elicited plants was harvested 4 days after the first elicitation, and dried as above.

For the N-partitioning analysis, both the locally elicited yRL and oRL were harvested 4 days after the first elicitation and flash-frozen in liquid N_2. After stalk elongation, the first stem leaf (S1) was harvested when it reached the source–sink transition stage.

For all three leaves, only the right leaf blade was harvested to standardize sampling and minimize changes in source–sink relationships arising from repeated sampling. The harvest time points of the S1 leaf differed depending on plant development. The first mature seed capsules were harvested at the day of opening: seeds were counted, weighed and analyzed for N content. For the kinetic analysis, plants received a ^{15}N pulse and were elicited as described above, and the locally elicited yRL was harvested at 0, 1, 3, 4 and 7 days after the first elicitation (Figure 1b). The sample size for all analyses was five.

Protein extraction and quantification

The TSP and RuBisCO LSUs and SSUs were extracted and quantified by Bradford assay and LC-MS^E, respectively, as described by Ullmann-Zeunert *et al.* (2012). The ^{15}N-incorporation of RuBisCO was determined with the Excel spreadsheet ProSIPQuant (Taubert *et al.*, 2011).

Metabolite extraction and quantification

Small metabolites were extracted as in Gaquerel *et al.* (2010) and analyzed by UPLC/UV/ToF-MS, using a Dionex RSLC system with a diode array detector (Dionex, http://www.dionex.com) and a Micro-ToF Mass Spectrometer (Bruker, http://www.bruker.com). Further details on instrument parameters and quantification are described in Appendix S1. Average mass spectra were extracted for ^{15}N-incorporations using the Excel spreadsheet ProSIPQuant (Taubert *et al.*, 2011), modified for small metabolites based on compound sum formulae.

Isotope ratio mass spectrometry analysis (IRMS)

The IRMS sample preparation, analysis and following calculations of total N content (% dry mass) and ^{15}N-incorporation were carried out as described in Meldau *et al.* (2012).

Statistical analysis

The R environment was used for statistical analysis (Team, 2009). For ANOVA and ANCOVA analyses, if the assumption of homoscedasticity of variances was violated or the residuals did not follow a normal distribution, response variables were transformed prior to the analyses using Box–Cox transformation (see Appendix S2). The Box–Cox lambda was estimated using Venables' and Ripley's MASS library for R. All ANOVA models were simplified to the minimum adequate model using Aikaike's information criterion (Ronchetti, 1985). For the correlation analysis (Figure 4a, heat maps) the data were imported into the environment and vectors containing the following variables were generated: N-rest protein μg mg^{-1}, N-RuBisCO LSU μg mg^{-1}, N-RuBisCO SSU μg mg^{-1}, N-nicotine μg mg^{-1}, N-CP μg mg^{-1} and N-DCS μg mg^{-1}. These vectors were pairwise correlated, calculating Kendall's τ coefficient (Kendall, 1938). In contrast to Pearson's correlation coefficient, Kendall's τ is more robust and not sensitive to the data distribution.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The dry mass and the absolute amount of nitrogen (N) of the shoot are not influenced by genotype.

Figure S2. Average leaf size produced by transgenic (ir*LOX3*, ir*MYB8*) and WT plants with and without the W + OS treatment.

Figure S3. Silencing of *LOX3* and *MYB8* alters the absolute pools of RuBisCO (a) and N-containing small metabolites (b) in leaves.

Figure S4. Reproductive timing and output produced by transgenic (ir*LOX3*, ir*MYB8*) and WT plants with and without the W + OS treatment.

Figure S5. Increased N investment into nicotine, CP and DCS is accompanied by a decreased N investment into RuBisCO.

Figure S6. The decrease of N investment into protein pools is greater than the amount of N required for the biosynthesis of the N-containing defense metabolites.

Figure S7. ^{15}N-incorporation in the yRL is not influenced by treatment or genotype.

Appendix S1. Supplemental procedures.

Appendix S2. Supplemental statistical information.

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Supplemental Figure Legends:

Figure S1: The dry mass and the absolute amount of nitrogen (N) of the shoot are not influenced by genotype.

Absolute N content of the shoot was calculated by multiplying the shoot N content (% dry mass) by the mass of the shoot (n=5).

Figure S2: Average leaf size produced by transgenic (ir*LOX3*, ir*MYB8*) and WT plants with and without the W+OS treatment.

Figure S3: Silencing of *LOX3* and *MYB8* alters the absolute pools of RuBisCO (a) and N-containing small metabolites (b) in leaves.

The absolute pool sizes were calculated by multiplying the concentration of the compounds by the leaf mass (n=5). LOQ=below limit of quantification; n.d.=not detectable.

Figure S4: Reproductive timing and output produced by transgenic (ir*LOX3*, ir*MYB8*) and WT plants with and without the W+OS treatment.

Average time until first day of flowering and first capsule maturation and average number of mature capsules per plant (a) and average mass per first seed capsule and total N of pooled seeds from the first seed capsule (b) were recorded. Absolute N content of capsules was calculated by multiplying the percentage N concentration by the mass of the pooled seeds of the first capsule (n=5). d.a.g= days after germination.

Figure S5: Increased N investment into nicotine, CP and DCS is accompanied by a decreased N investment into RuBisCO.

Same data displayed in Figure 4a but without TSP.

Figure S6: The decrease of N investment into protein pools is greater than the amount of N required for the biosynthesis of the N-containing defense metabolites.

N-investments were calculated by multiplying the proportion of N present in each compound with the concentration of the compound (n=5).

Figure S7: ^{15}N-incorporation in the yRL is not influenced by treatment or genotype.

Figure S1

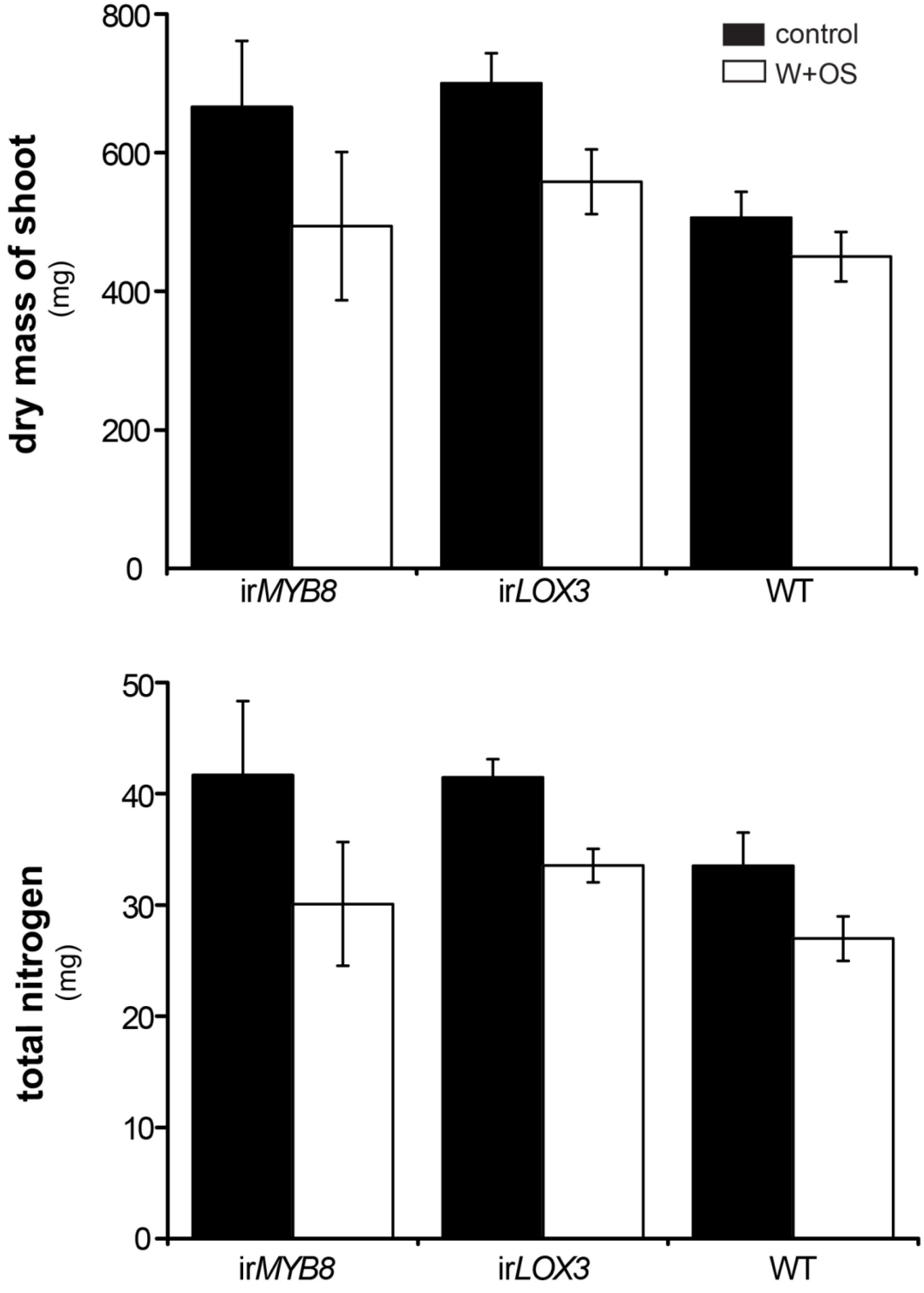

800
600
400
200
0
dry mass of shoot
(mg)
control
W+OS
irMYB8
irLOX3
WT
50
40
30
20
10
0
total nitrogen
(mg)
irMYB8
irLOX3
WT

Figure S2

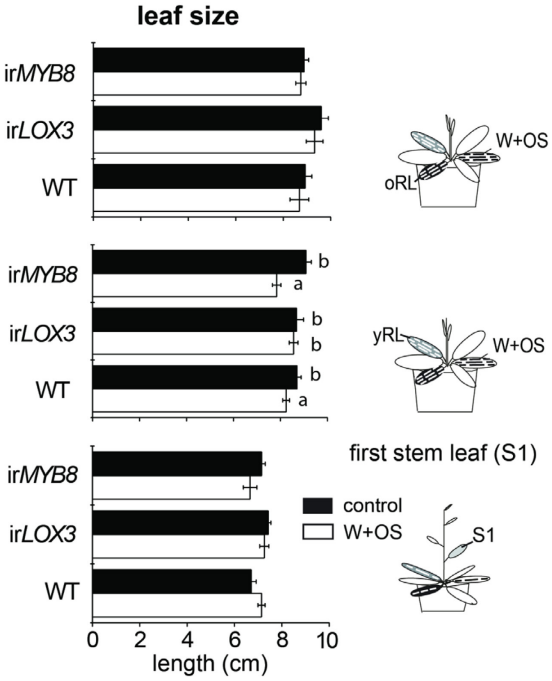

leaf size
irMYB8
irLOX3
WT
W+OS
oRL
b
a
b
b
b
a
yRL
W+OS
first stem leaf (S1)
control
W+OS
S1
0
2
4
6
8
10
length (cm)

Figure S3

a)

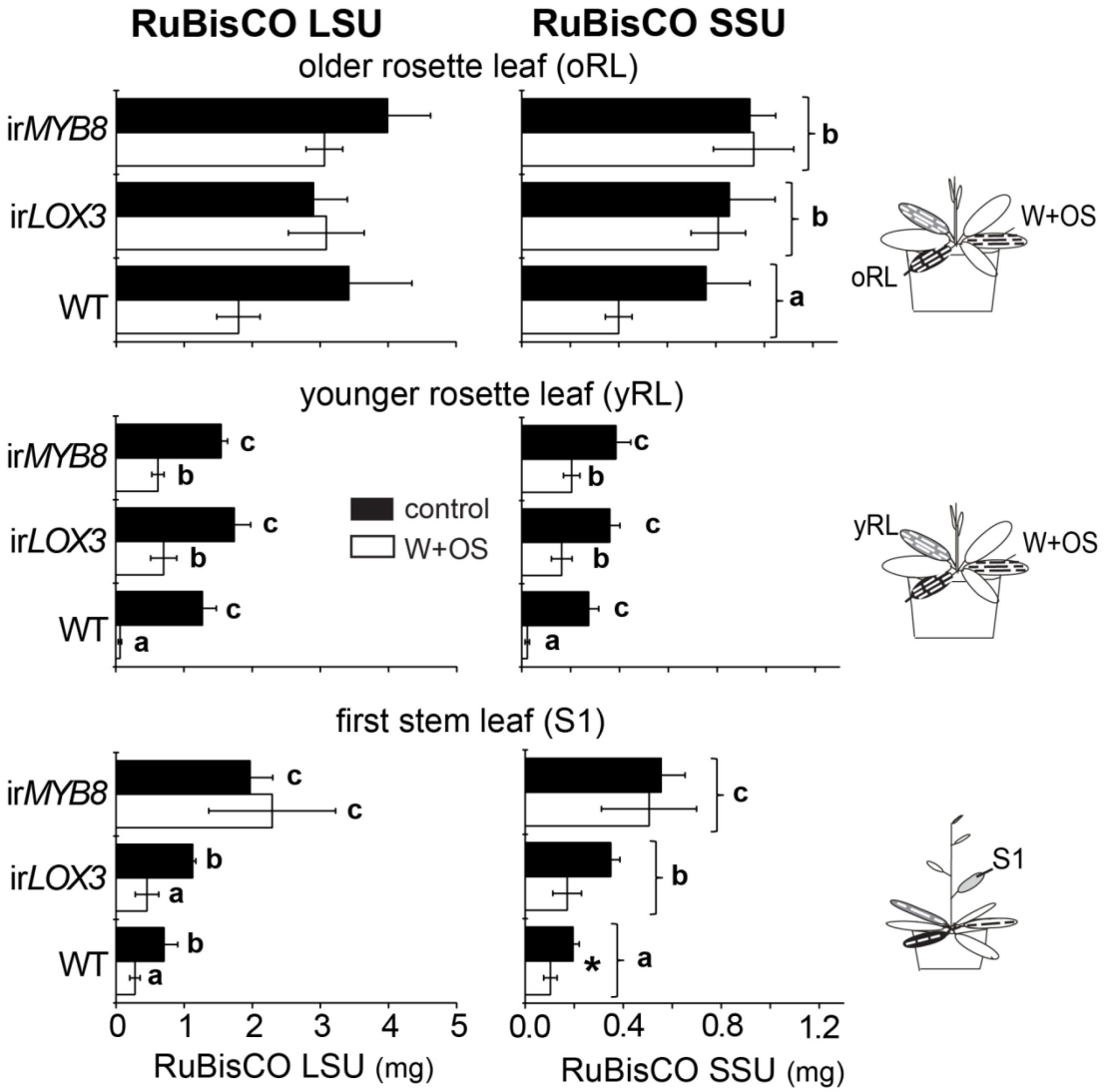

RuBisCO LSU
RuBisCO SSU
older rosette leaf (oRL)
younger rosette leaf (yRL)
first stem leaf (S1)
irMYB8
irLOX3
WT
control
W+OS
oRL
yRL
S1
RuBisCO LSU (mg)
RuBisCO SSU (mg)

Figure S3 continued

b)

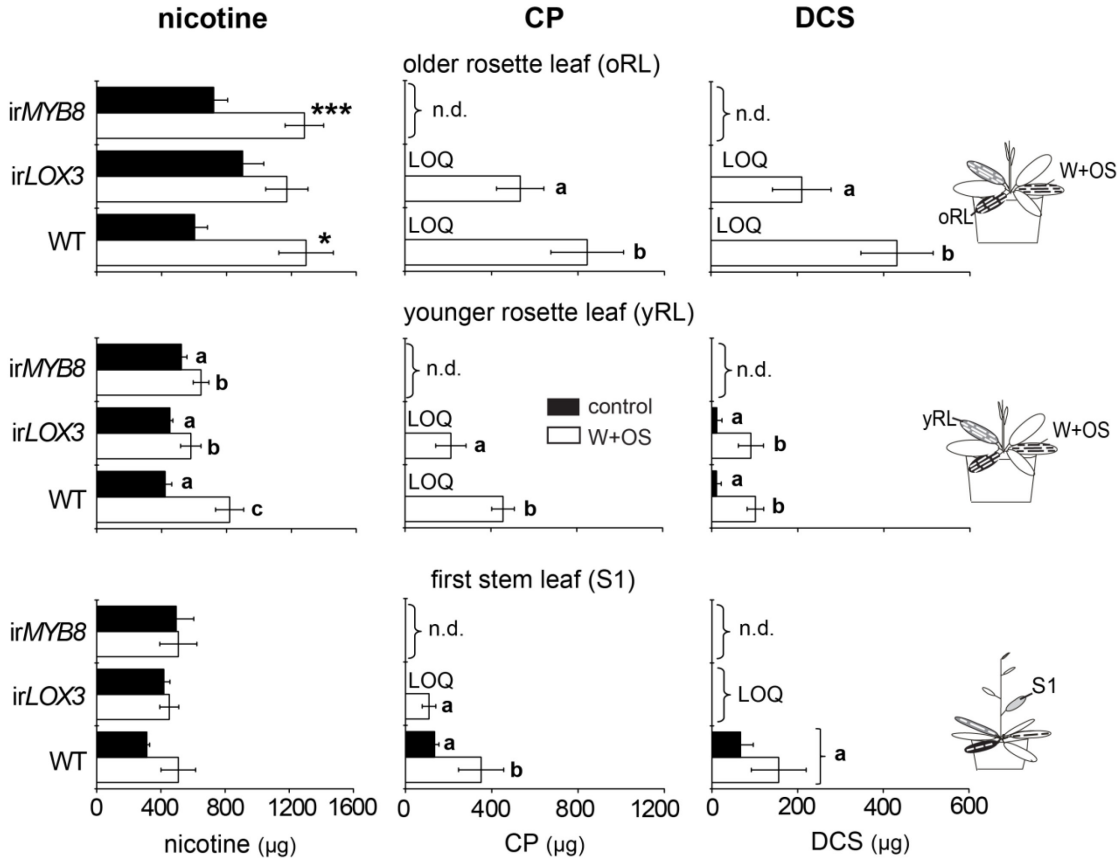


Figure S4

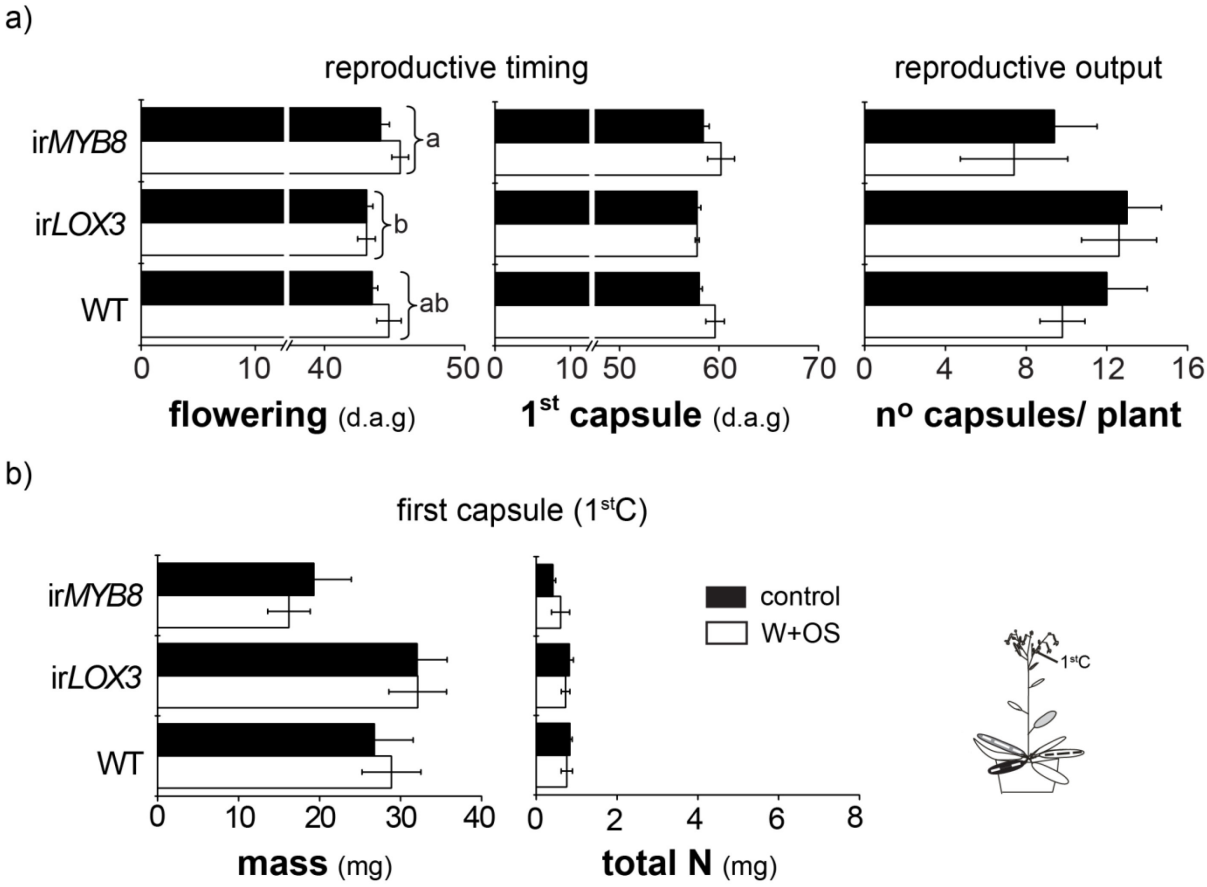

a)
reproductive timing
reproductive output
irMYB8
irLOX3
WT
a
b
ab
0 10 40 50
0 10 50 60 70
0 4 8 12 16
flowering (d.a.g)
1st capsule (d.a.g)
nº capsules/ plant
b)
first capsule (1stC)
irMYB8
irLOX3
WT
0 10 20 30 40
mass (mg)
control
W+OS
0 2 4 6 8
total N (mg)
1stC

Figure S5

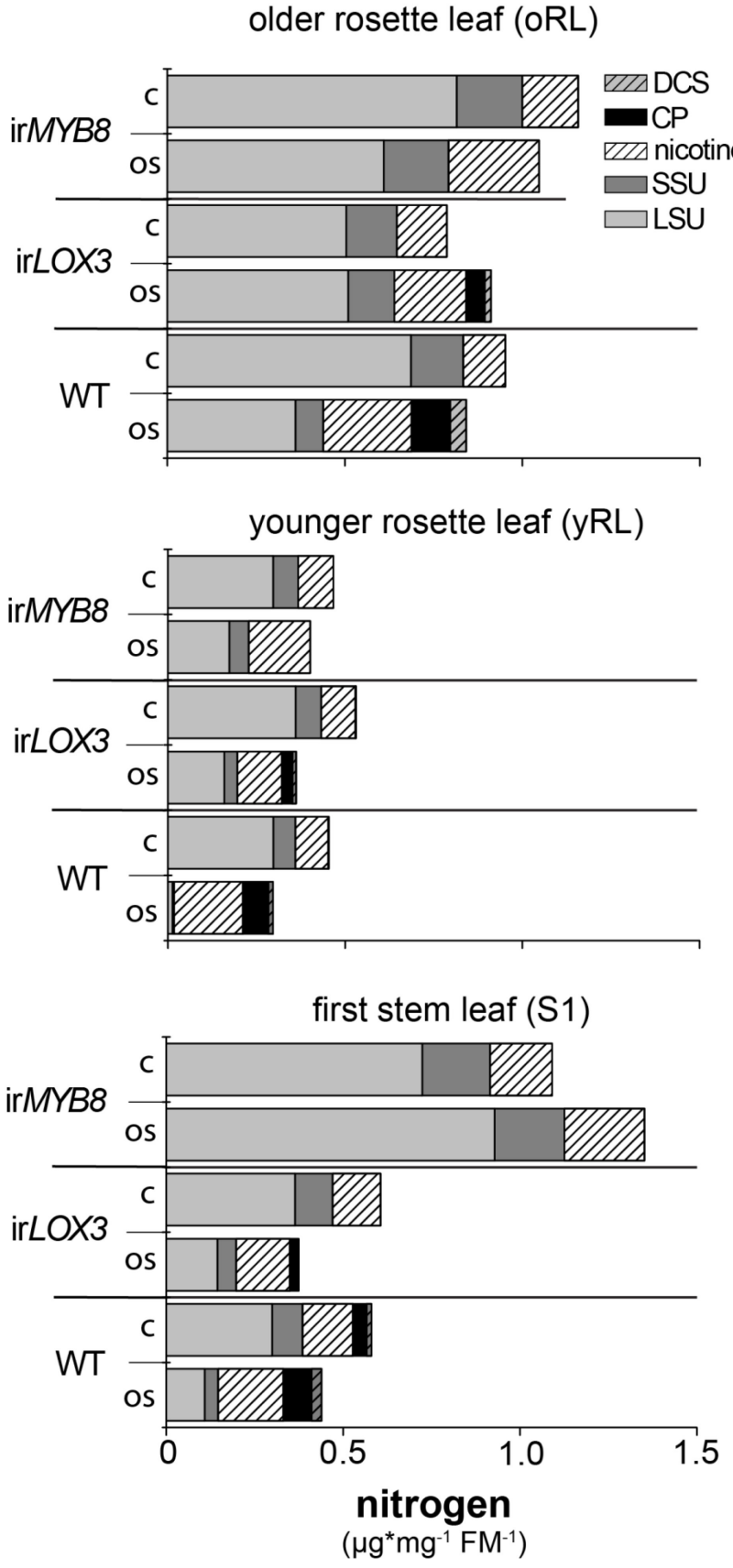

older rosette leaf (oRL)
younger rosette leaf (yRL)
first stem leaf (S1)
irMYB8
irLOX3
WT
c
os
DCS
CP
nicotine
SSU
LSU
0
0.5
1.0
1.5
nitrogen
(µg*mg-1 FM-1)

Figure S6

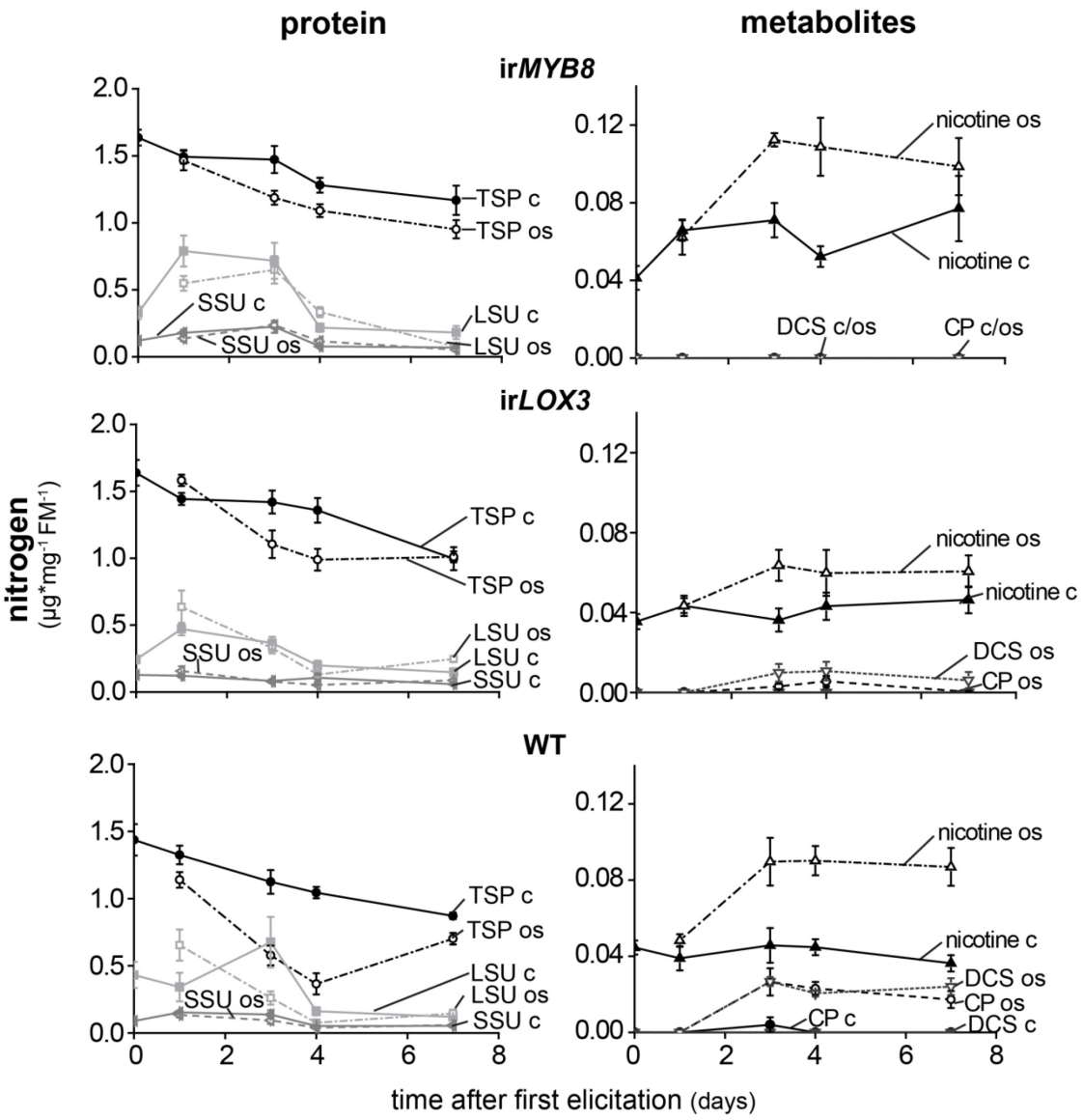
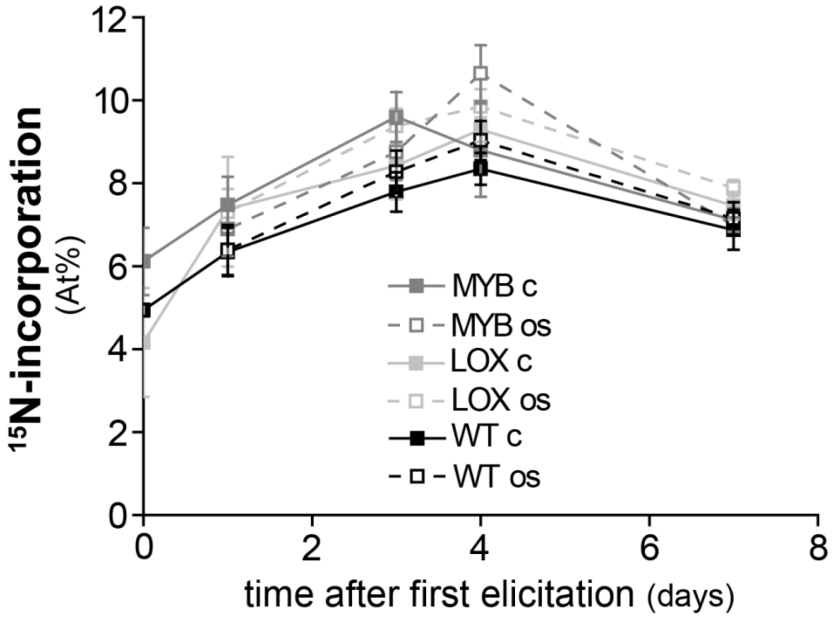

protein
metabolites
irMYB8
irLOX3
WT
nitrogen (µg*mg-1 FM-1)
TSP c
TSP os
SSU c
SSU os
LSU c
LSU os
nicotine os
nicotine c
DCS c/os
CP c/os
DCS os
CP os
CP c
DCS c
time after first elicitation (days)

Figure S7


12
10
8
6
4
2
0
^{15}N-incorporation (At%)
0
2
4
6
8
time after first elicitation (days)
MYB c
MYB os
LOX c
LOX os
WT c
WT os

Appendix S1: Supplemental Statistical Information

For the following response variables, the assumption of homoscedasticity of variances was violated or the residuals did not follow a normal distribution; they were transformed
using Box-Cox transformation prior to the analyses.
Fig. 3: total amount of TSP in S1 leaves; Fig. S3a total amounts of RuBisCO SSU in all leaves, RuBisCO LSU in yRL and S1 leaves; Fig. 3b: total amounts of nicotine in oRL and yRL; total amounts of DCS in yRL.

Supplemental Figures:

Figure S1: ANOVA, a) Treatment, $F_{1,28} = 4.68$, $p = 0.039$; b) Treatment, $F_{1,28} = 7.28$, $p = 0.011$.

Figure S2: a) ANOVA, oRL: non-significant (n.s.); yRL: $F_{1,28} = 14.60$, $p = 6.762*10^{-4}$; S1: n.s. (non-transformed data), b) ANOVA, mass: n.s.; total N: n.s.

Figure S3: Asterisks indicate differences among treatments (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). Letters represent significant differences in the minimum adequate model. (ANOVA, a) RuBisCO LSU: oRL: n.s.; yRL: $F_{2,27} = 78.25$, $p = 5.81*10^{-12}$; S1: $F_{2,26} = 30.084$, $p = 1.719*10^{-7}$; RuBisCO SSU: oRL: Line: $F_{1,28} = 7.91$, $p = 0.009$; yRL: $F_{2,27} = 44.65$, $p = 2.74*10^{-9}$; S1: Line: $F_{2,26} = 13.76$, $p = 8.37*10^{-5}$; Two sample T-test: ir*LOX3*: $p = 0.035$, WT: $p = 0.040$; b) nicotine: oRL: Treatment: $F_{1,28} = 27.77$, $p = 1.32*10^{-5}$; Two sample T-test: irLOX3: $p = 0.053$; ir*MYB8*: $p = 0.009$; WT: $p = 0.020$; yRL: $F_{2,27} = 14.69$, $p = 4.82*10^{-5}$; S1: n.s.; CP: oRL: $F_{2,27} = 44.537$, $p = 2.81*10^{-9}$; yRL: $F_{1,28} = 77.69$, $p = 6.31*10^{-12}$; S1: $F_{2,25} = 31.89$, $p = 1.32*10^{-7}$; DCS: oRL: $F_{2,27} = 36.09$, $p = 2.35*10^{-8}$; yRL: $F_{2,27} = 24.64$, $p = 8.15*10^{-7}$; S1: Line: $F_{2,25} = 11.66$, $p = 2.65*10^{-4}$).

Figure S5: n = 121; ANOVA, Line:Treatment:Time: $F_{2,109} = 0.201$, $p = 0.98$

Supplemental Procedures

Detection and quantification of small N-containing metabolites

Three microliters of extract were separated on a Dionex RSLC system (Dionex, Sunnyvale, USA) equipped with a Dionex Acclaim 2.2um 120A 2.1x150 mm column, under the following gradient: initial composition: 95% A (deionized water, 0.1% [v/v] acetonitrile [HPLC grade, Merck, Darmstadt, Germany], and 0.05% FA), 5% B (acetonitrile and 0.05% FA); 0 to 29 min linear gradient to 25% B; 29 to 32 min linear gradient to 80% B; isocratic for 6 min. The flow rate was 300 µL/min and the instrument was equilibrated at 95% A for 6 min after runs. Eluted compounds were detected using a Diode Array Detector and subsequently by MicroToF Mass Spectrometer (BrukerDaltonik, Bremen, Germany), with the ionization conditions described in Gaquerel *et al.*(2010).

Quantification was based on the UV trace at the wavelengths 254 nm (nicotine) and 320 nm (CP and DCS) using an external standard curve (20, 40, 80, 160, 320, 480 and 640 ng on column for nicotine and 10, 20, 40, 80 and 160 ng on column for CP and DCS). Extracted ion chromatograms for each compound were generated from raw data using Data Analysis software (BrukerDaltonik, Bremen, Germany).

Manuscript III

SHORT COMMUNICATION

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Silencing ribulose-1,5-bisphosphate carboxylase/ oxygenase expression does not disrupt nitrogen allocation to defense after simulated herbivory in *Nicotiana attenuata*

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Abbreviations: N, nitrogen; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; *asRUB*, *N. attenuata* line silenced for RuBisCO expression using an antisense construct; OS, oral secretions; TSP, total soluble protein; CP, caffeoyl-putrescine; DCS, dicaffeoyl-spermidine; LSU, RuBisCO large subunit; SSU, RuBisCO small subunit; W+OS, wounding and oral secretion elicitation; yRL, young rosette leaf; LC-MS^E, liquid chromatography coupled with a time-of-flight mass spectrometer with alternating low energy and high energy scan acquisition; UPLC/UV/ToF-MS, ultrahigh pressure liquid chromatography coupled with an ultra-violet diode array detector and a time-of-flight mass spectrometer

Ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO) is the most abundant protein on the planet and in addition to its central role in photosynthesis it is thought to function as a nitrogen (N)-storage protein and a potential source of N for defense biosynthesis in plants. In a recent study in the wild tobacco *Nicotiana attenuata*, we showed that the decrease in absolute N invested in soluble proteins and RuBisCO elicited by simulated herbivory was much larger than the N-requirements of nicotine and phenolamide biosynthesis; ^{15}N flux studies revealed that N for defensive phenolamide synthesis originates from recently assimilated N rather than from RuBisCO turnover. Here we show that a transgenic line of *N. attenuata* silenced in the expression of RuBisCO (*asRUB*) invests similar or even larger amounts of N into phenolamide biosynthesis compared with wild type plants, consistent with our previous conclusion that recently assimilated N is channeled into phenolamide synthesis after elicitation. We suggest that the decrease in leaf proteins after simulated herbivory is a tolerance mechanism, rather than a consequence of N-demand for defense biosynthesis.

Plant defense strategies against herbivore attack are generally categorized into resistance and tolerance strategies[1] and several theories have been proposed to explain these strategies.[2] One of the best experimentally supported theories is the optimal defense theory which states that defenses are adaptive but have allocation costs, since resources can either be invested into growth and fitness or into defense.[3-5] This growth-defense trade-off can be minimized by using inducible defenses which are only produced upon attack.[2,6,7] Consequently, plants optimize their allocation of growth-limiting resources to resistance and tolerance strategies or to growth and reproduction according to herbivore pressure.[8] These growth-defense trade-offs are thought to be more stringent in the case of limited resources such as nitrogen (N) which affects the growth and fitness of plants and of the herbivores that feed on them. At the molecular level, components needed for growth such as sugars, amino acids and proteins, may also serve as a direct source of carbon and N for the biosynthesis of defensive chemicals.[9-12] It has been hypothesized that one major source of N for the production of defensive metabolites may be proteins,[9,10] and among these one potentially important N source is the photosynthetic protein ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO), which is the most abundant protein in foliar tissues[13-15] and is thought to serve as a N storage protein.[16] This hypothesis has rarely been tested at the level of individual compounds, due to technical difficulties in comparing the allocation into different classes of molecules such as large proteins and small molecular weight defensive metabolites (e.g., alkaloids, polyamine conjugates) in a common currency.

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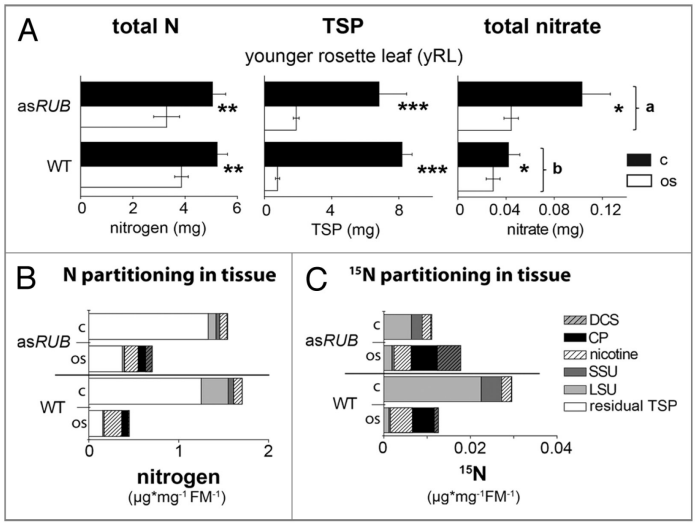

Figure 1. Silencing of ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO) does not alter major foliar N pools but decreased N and ^{15}N investment into RuBisCO is accompanied by an increase in defense metabolite investment after OS-elicitation of sink leaves (yRL). **a)** RuBisCO-silenced lines (as*RUB*) have similar total N pools and total soluble protein as wild-type (WT), but silencing causes increases in total nitrate pools. Asterisks indicate differences among treatments in a two-way ANOVA (**: $P \leq 0.01$; ***: $P \leq 0.001$). Letters represent significant differences found using the minimum adequate model (n = 5). as*RUB* plants show increased N-(**b**) and ^{15}N-(**c**) partitioning to dicaffeoyl-spermidine (DCS). N/^{15}N investment in residual TSP (TSP – (SSU + LSU)), RuBisCO large (LSU) and small (SSU) subunit, nicotine, caffeoyl-putrescine (CP) and DCS in the yRL was calculated by multiplying the proportion of N/^{15}N in each compound with the concentration of the compound for each leaf. Concentrations of each analyte were measured as described in the text and total pools were calculated based on leaf mass. Plants were elicited as described in text and the yRL was harvested 4 d after the first wound and OS elicitation. Note that ^{15}N was not measured in the residual TSP. c – control, OS – treatment with wound and oral secretions of *Manduca sexta*, TSP – total soluble protein

One major resistance mechanism of the wild tobacco *Nicotiana attenuata*, a model plant for the study of plant-herbivore interactions, is the production of the N-containing defense metabolites nicotine and phenolamides, caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS). In a recent study, we used ^{15}N pulse-labeling and a novel LC-MS^E quantification method for proteins to quantify growth-defense trade-offs using N as a common currency after simulated herbivory using oral secretions (OS) of the specialist tobacco hornworm *Manduca sexta*.[17] Our results showed that although OS-elicited *N. attenuata* plants show large decreases in total soluble protein (TSP) and RuBisCO pools, the N required for synthesis of the metabolically dynamic defensive phenolamides, caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS), does not come from the putative storage protein RuBisCO, but likely from recently assimilated N.

Here we use a previously described transgenic line of *N. attenuata* stably silenced in the expression of RuBisCO using an antisense construct,[18] hereafter called as*RUB* plants. At the whole plant level, these as*RUB* plants were shown to have a reduced accumulation of the RuBisCO protein, a 25% reduction in photosynthesis rates, and stalk lengths were transiently shorter, but they eventually attained the heights of empty vector-transformed plants.[18] In the present study, we quantify N based growth-defense trade-offs in as*RUB* plants and compare them to WT plants in the same experimental design used in the previous study.[17] Briefly, soil-grown plants were pulse-labeled with 5.1 mg of ^{15}N, supplied as $K^{15}NO_3$, 3 d before repeated wounding and elicitation with 1:5 diluted *M. sexta* OS in glasshouse conditions. The oldest sink, youngest source and transition rosette leaves were wounded 1x per day for three consecutive days and on each day 10 µL of diluted OS was added immediately to the wound. Four days after the first wounding and OS elicitation (W+OS), the locally elicited sink leaf (hereafter referred to as young rosette leaf, yRL) was harvested and analyzed. Leaf total N was measured by isotope ratio mass spectrometry (IRMS), TSP was measured by the Bradford assay, nitrate was measured following ref.[19] and absolute pools and ^{15}N incorporation into RuBisCO and nicotine and phenolamides were measured respectively by LC-MS^E and UPLC/UV/ToF-MS as described in ref.[17]

Our data demonstrates that as*RUB* plants have similar whole leaf N accumulation and TSP pools as WT plants, with a slight decrease of whole leaf N and a sharp decrease of TSP after OS elicitation (**Fig. 1A**). This suggests that even though as*RUB* lines have decreased accumulation of the large subunit of RuBisCO (LSU, see **Table 1**), their TSP pools follow the same dynamics as WT plants after OS elicitation. Additionally, it suggests that N not invested in RuBisCO in as*RUB* is at least partially diverted to other soluble proteins. Interestingly, although as*RUB* are silenced for accumulation of LSU, they invest similar amounts of N into CP and significantly greater amounts into DCS after OS elicitation compared with WT (**Fig. 1B**, **Table 1**). Investment of ^{15}N follows the same general pattern as N investment, with significantly higher amounts of ^{15}N allocated to DCS in OS-elicited as*RUB* lines compared with WT (**Fig. 1C**). These findings are consistent with our previous conclusions that recently assimilated N is invested into phenolamide production after *M. sexta* OS elicitation and that, at least in the case of CP and DCS, the N for the synthesis of defense metabolites is not derived from RuBisCO degradation (see **Table 1** for results of the statistical analysis and absolute pools of individually measured metabolites). It is also striking that though the total amounts of N and TSP are similar in both lines, as*RUB* leaves accumulate significantly more nitrate than WT leaves; after simulated herbivory the amounts of nitrate decrease to the same extent in both lines (**Fig. 1**). In view of

Table 1. Absolute pools of RuBisCO LSU, SSU, Nicotine, CP and DCS in µg measured in the yRL of WT and as*RUB* lines, final stalk size in cm and maturation time of the first stalk leaf (S1, d.a.g. = days after germination); and F – and *P* = values for 2 factor ANOVAs for each compound. Absolute pools were calculated by multiplying the concentration of the compounds by the leaf mass (n = 5) as described in 17. *LOQ* = below limit of quantification. All values were box-cox transformed prior analysis to meet test assumptions, except for stalk size.

	Average (SE)				ANOVA F – and p-values					
					Genotype		Treatment		interaction	
	WT c	WT os	asRUB c	asRUB os	F	p	F	p	F	p
LSU [µg]	1269 (204)	61.6 (24.2)	481.4 (176.5)	73.6 (21.8)	9.76	**0.007**	39.9	**< 0.001**	9.20	**0.008**
SSU [µg]	273.7 (41.5)	22.9 (8.7)	206.4 (55.3)	27.7 (4.4)	0.55	0.469	86.78	**< 0.001**	2.039	0.174
Nicotine [µg]	421.4 (41.6)	819.5 (86.9)	356.1 (36.5)	622.01 (99.5)	3.38	0.085	19.93	**< 0.001**	0.50	0.489
CP [µg]	LOQ	456.3 (52.7)	LOQ	524.5 (67.6)	0.04	0.851	6.89E+19	**< 0.001**	0.04	0.851
DCS [µg]	11 (11)	101.6 (19.4)	74.6 (34.5)	566.1 (71.6)	23.06	**< 0.001**	42.62	**< 0.001**	0.49	0.494
stalk [cm]	52.2 (0.7)	48.7 (1.0)	51.8 (0.6)	50.1 (1.1)	0.35	0.563	8.34	**0.01**	1.00	0.332
S1 [d.a.g.]	36.6 (0.4)	38.8 (0.9)	37.4 (0.2)	37.6 (0.5)	0.04	0.840	4.44	0.051	3.22	0.092

the conclusions outlined above, it's tempting to speculate that the higher investments into DCS in the transgenic line are derived from this recently acquired N-pool. By using recently assimilated N, the plants might be able to react more quickly to herbivore attack, by rapidly increasing defense biosynthesis and decreasing and remobilizing N invested in proteins, and together these responses could function to anticipate the readjustments required after the loss of resources.[20,21]

The total amounts of nitrate measured in as*RUB* plants are much smaller than the amounts of DCS that accumulate after simulated herbivory (**Fig. 1A**, **Table 1**), but this may be due to the high turnover rate of the nitrate pool, and a single time-point measurement would not reflect a constant higher N-supply from this pool, which could be used for DCS biosynthesis. Our results are in contrast to earlier publications which suggest that RuBisCO is primarily a storage protein, providing N for defense metabolite production after herbivory[16]; and also to studies in *N. tabacum* silenced for RuBisCO expression, where RuBisCO-silenced plants had higher nitrate levels than WT but invested less into N-containing metabolites (nicotine), due to carbon limitation.[22] In the present study, constitutive and OS-induced nicotine levels are slightly reduced in as*RUB* leaves, but the difference between the genotypes was not significant (**Fig. 1B**, **Table 1**). Similarly, though the nicotine levels were significantly increased after OS elicitation in both as*RUB* and WT leaves, the ^{15}N incorporation into nicotine was overall low and did not differ significantly between the transgenic line and WT (**Fig. 1C**). Thus in *N. attenuata* we cannot find a direct growth-defense trade-off based on nicotine after simulated herbivory with OS from a specialist herbivore which is adapted to high levels of nicotine. It is noteworthy that these previous studies in cultivated tobacco silenced for RuBisCO have focused on the effects of carbon or N limitations on growth-defense trade-offs, but did not analyze the effects of herbivory on the allocation to growth and N-based defenses.[22,23] Therefore, it is possible that in the absence of herbivore attack, resources are redirected to metabolic functions other than defense, to allow plants to better tolerate growth under carbon – or N-limited conditions. Another possible explanation is that herbivory-induced

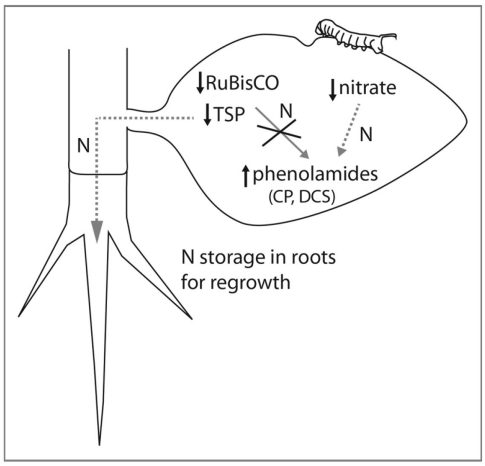

Figure 2. Hypothetical model of nitrogen (N)-flow after herbivory. Total pools of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and total soluble proteins (TSP) decrease after herbivory. N originating from these proteins is not used for the biosynthesis of rapidly induced N-containing defensive phenolamides (caffeoyl-putrescine,CP and dicaffeoyl-spermidine, DCS), but probably redirected to the root; while phenolamides are synthesized from recently assimilated N, possibly nitrate.

increases in plant N allocation to defense are stronger in *N. attenuata* compared with cultivated tobacco, since the former is a pioneer species, adapted to growth in a desert environment with high herbivore pressure by both nicotine-tolerant specialists and nicotine-sensitive generalist herbivores. In this context, we suggest that after elicitation by OS of the nicotine-tolerant *M. sexta*, *N. attenuata* invests N into the rapidly inducible phenolamides which have been shown to be effective against this herbivore,[24,25] while maintaining high nicotine levels which are effective against generalist herbivores.

Based on these results we propose that the OS-elicited dramatic decline in soluble proteins in *N. attenuata* is mainly a

tolerance mechanism, possibly a strategy to decrease the digestibility of leaf tissue to attacking herbivores or of facilitating the reallocation of N to other plant parts.[8,12] We speculate that protein-derived N is mainly reallocated to roots, thus enabling regrowth after herbivore attack (**Fig. 2**) as previously shown for carbon in wild tobacco and tomato: where newly assimilated carbon was transported to the roots after simulated leaf herbivory, probably to be used for post-herbivory re-growth, instead of being transported to the young leaves.[26,27]

Furthermore, our results suggest that the N-demand for defense metabolite production itself does not seem to be the direct signal leading to a major switch in metabolism after herbivory which results in a decline in growth functions and increased investment in defenses, since the transgenic line used here (as*RUB*) had similar or even higher investments in N-containing defenses as WT plants, despite decreased accumulation of the most abundant foliar protein in plants.

These findings are also consistent with the idea that the production of defense metabolites is adaptive: if plants have more free nutrients (in this case, nitrate) available they have a higher capacity for the rapid production of N-containing defense metabolites to protect their valuable photosynthetically active tissues. In the case of *N. attenuata* as*RUB* plants, these additional investments in defenses after OS elicitation are not more costly for the plant, since OS-elicited as*RUB* plants were not significantly different from WT in growth parameters measured (final stalk length, maturation of the first stem leaf, S1; **Table 1**). This is consistent with a previous study on this as*RUB* line which showed that despite decreased photosynthetic rate and lower levels of RuBisCO which affected initial growth rates, these plants eventually achieved the same stalk height as WT plants, possibly due to increased activation of RuBisCO which compensates for lower foliar pools of this protein.[18,28] It is unclear however, what effects the silencing of RuBisCO would have on plant growth after longer periods of herbivore damage and also in the case of intra-specific competition. It would be interesting to test whether the proposed tolerance mechanism above, based on N reallocation from proteins, is affected by the silencing of this important photosynthetic protein in the long-term in more natural settings.

Overall, our data suggest that there is no direct resource-based trade-off between N-demand for defense metabolite production and a decrease in protein content within the leaf, but that the regulation of trade-offs between defense metabolite production and plant development and tolerance is rather based on whole-plant signaling mechanisms which remain to be elucidated. The availability of free N (such as nitrate) might also play a role which needs to be further investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Manuscript IV

Plant-mediated plasticity in emission of an aggregation pheromone provides a reliable signal for a native tobacco seed feeder to locate an unreliable host plant in the field

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Abstract

The use of aggregation pheromones by phytophagous insects is thought to provide information-based solutions to the challenge of finding unpredictable host plants, but this premise is rarely tested. *Corimelaena extensa* is a common hemipteran seed feeder of the perennial desert tobacco, *Nicotiana obtusifolia*, yet it consistently forms large aggregations on the unpredictable fire-chasing annual, *N. attenuata*. We hypothesized that *C. extensa* mitigates the costs of finding *N. attenuata* by using a pheromone as a reliable host cue, and that these costs are compensated by higher host-dependent fitness. We then identified and synthesized the aggregation pheromone - (5Z, 8Z)-tetradeca-5,8-dienal - and used the synthetic product to test its adaptive value in the colonization of both host plants. We found that *C. extensa* emits higher amounts of the pheromone and has higher fitness when feeding on *N. attenuata*. We conclude that this newly discovered aggregation pheromone allows the insect to find the highly unpredictable host on which it realizes the greatest fitness.

Introduction

Phytophagous insects emerging from overwintering sites in spring must disperse to find new host plants, which are frequently heterogeneously distributed in the landscape. Plant-derived infochemicals, such as plant volatile blends, are involved in long distance recognition of host plants (Visser 1986; Bruce *et al.* 2005; Halitschke *et al.* 2008), but in many species of phytophagous insects aggregations of conspecifics on hosts are also mediated by active chemical communication among individuals, through use of aggregation pheromones which attract conspecifics of both sexes or of the same sex as the emitter (reviewed in Wertheim *et al.* 2005). Although many studies have characterized aggregation pheromones in a mechanistic and applied perspective in a variety of insect taxa, there are still few studies of the evolutionary ecology of these pheromones in a cost-benefit framework to understand their adaptive value (Millar 2005; Wertheim *et al.* 2005). One of the best studied systems are the interactions of many species of bark beetles (Coleoptera: Scolytinae) and their conifer hosts, in which beetles use a concerted attack mediated by aggregation pheromones that overcomes the host's defenses allowing them to inoculate the trees with

their symbiotic fungi, which provide nutrition for their larvae (reviewed in Wood 1982; Byers 1989) and recent studies have shown additional layers of complexity in the interaction between host plant chemicals with beetles and symbiotic fungi and bacteria which can conditionally increase or decrease the costs and benefits of each member (reviewed in Raffa 2013). Potential benefits arising from pheromone-mediated aggregative behavior in non-social insects include more efficient resource use and overcoming of host defenses, improved abiotic conditions, better mate finding, and more effective protection against natural enemies. However, this behavior can also incur costs such as increased competition for food and mates, increased apparency to natural enemies, and also the costs of investing in pheromone production for the emitting insects (Wertheim *et al.* 2005; Johansson & Jones 2007).

True bugs (Hemiptera: Heteroptera) are one of the most diverse hemimetabolous insect orders, about half of which are phytophagous and many are important crop pests(Weirauch & Schuh 2011). They are characterized by well-developed scent glands in both adults and nymphs and there is evidence that aggregation in true bug adults and nymphs is often mediated by pheromones (Aldrich 1988; Millar 2005). But despite a great advance in characterization of chemicals produced by true bugs and also in their application for biological control, the roles of these compounds in insect behavior and their adaptive value is still poorly understood in most species (Millar 2005). Furthermore, the understanding of host selection in phytophagous insects has been extensively studied in the context of adults choosing host plants that maximize juvenile fitness (the "preference-performance hypothesis") and it has been mostly studied in insects whose adults do not feed at the oviposition site and have limited larval mobility, such as Lepidoptera. But host selection in true bugs is likely to be strongly affected both by optimal foraging and by optimal oviposition choices since adults often feed on the same plants as their offspring, frequently show communal feeding behavior and juveniles can be more mobile (Mayhew 1997; Scheirs & Bruyn 2002; Martinez *et al.* 2013). In this study we investigate a seed-feeding true bug that colonizes two wild tobacco species in the Great Basin Desert (USA) and shows characteristics of a pheromone-mediated aggregation. We identified and synthesized the aggregation pheromone and used the synthetic product to test the adaptive value of this infochemical in the colonization of both host plants.

The true bug *Corimelaena extensa* Uhler (Hemiptera: Thyreocoridae) is a common florivore and seed feeder of wild tobacco (*Nicotiana* spp., Solanaceae), in the southwestern

USA (Lung & Goeden 1982; McPherson 1982). Both adults and juveniles of Thyreocorid bugs aggregate and feed on the floral parts of their host plants and *Corimelaena* spp. feeding has been shown to significantly decrease seed set and viability of the coyote tobacco, *Nicotiana attenuata* Torr. ex. Watts (Baldwin & Morse 1994; Baldwin *et al.* 1997; Karban 1997), but little else is known of the ecology of these insects and their interactions with their tobacco hosts. In the Great Basin Desert, adult *C. extensa* emerge from yet unknown overwintering sites and colonize the perennial *N. obtusifolia* (Syn. *N. trygonophylla*, Dunal) in spring (April-May). But it's on the post-fire annual *N. attenuata* that these insects consistently form large feeding and mating aggregations, synchronized with the peak in this host's flowering phenology (May-June). *N. obtusifolia* is a perennial shrub that occurs in small populations on rocky outcrops which can persist for many growing seasons and typically flower between March and October, while *N. attenuata* is an annual which flowers between May and August and grows mainly as large populations that germinate synchronously from long-lived seed banks after fires in sagebrush and pinyon-juniper ecosystems (Fig. 1). Post-fire populations can persist for up to 3 growing seasons after fires, however small populations may persist for longer periods in isolated washes or as a roadside weed (Wells 1959; Lynds & Baldwin 1998). These differences in host plant life history strategies, namely shorter flowering phenology and population persistence over multiple growing seasons (years), would effectively make *N. attenuata* a less apparent, and therefore less reliable, host plant for insects emerging and dispersing from overwintering sites in spring. Due to the greater costs associated with increased dispersal (e.g.: higher mortality risk, higher energy expenditure and opportunity costs of delayed reproduction) (Rankin & Burchsted 1992) we expected that *C. extensa* would rely more heavily on *N. obtusifolia* as a host, since the fire-chasing annual *N. attenuata* represents a resource which is scattered in space and time; however the opposite is observed in native populations and *C. extensa* forms larger aggregations on *N. attenuata* (Fig. 1).

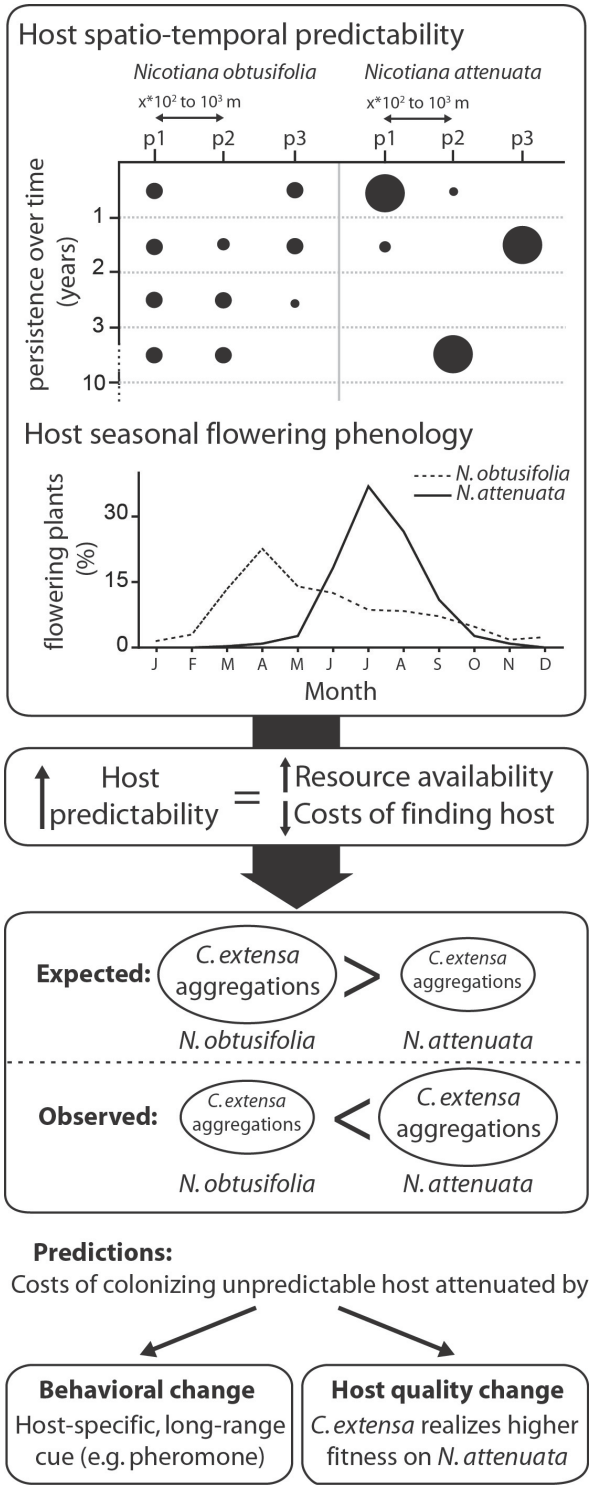

Figure 1. Overview of the study system and conceptual framework for host plant colonization by *Corimelaena extensa.* Distances between patches (p1, p2, etc) of the host plants *Nicotiana obtusifolia* and *N. attenuata* is similar, but irregular post-burn *N. attenuata* patches tend to be larger than *N. obtusifolia* patches. The hosts also differ in their persistence over the years and flowering phenology. A host with higher predictability is assumed to provide more resources and to diminish dispersal costs (e.g., energy and time investment) of host finding. Therefore we expect *C. extensa* to form larger aggregations on *N. obtusifolia*, but the opposite is observed in the field. We predict that the colonization of *N. attenuata* by *C. extensa* is mediated by use of specific and reliable cues, such as a pheromone, and through changes in host quality which lead to higher fitness on this host, mitigating the costs of host finding.

Based on these observations, we hypothesized that *C. extensa* mitigates dispersal costs by using specific and reliable long range cues to locate *N. attenuata* and engage in aggregation behavior. And since insect-produced cues are thought to be more reliable indicators of herbivore presence than plant-produced cues (Vet & Dicke 1992), we hypothesized that the cue is likely a long-range volatile pheromone ("Behavioral change", Fig. 1). We also hypothesized that the costs of finding the less apparent host and of investing in specific signaling behavior are compensated by higher insect fitness on this host compared to the more predictable *N. obtusifolia* ("Host quality change", Fig. 1). In this study we show that male adult *C. extensa* act as pioneers on *N. attenuata*, but not on *N. obtusifolia*. We further demonstrate through structural elucidation and *de novo* synthesis of a candidate volatile compound and manipulative field assays with the synthetic product, that the field colonization of the unpredictable *N. attenuata* by *C. extensa* is facilitated by an aggregation pheromone, (5Z, 8Z)-tetradeca-5,8-dienal, emitted by pioneer males. We also show that this pheromone is preferentially emitted on *N. attenuata* compared to the perennial *N. obtusifolia* and that *C. extensa* adults realize higher fecundity and nymphs have faster development time on the annual host. By manipulating insect density on both hosts, we show that the fitness benefits are increased by communal feeding on *N. attenuata*, suggesting a selection pressure favoring higher reliance on the aggregation pheromone on this host. We conclude that host plant identity mediates the phenotypic plasticity in the emission of this newly discovered aggregation pheromone and allows the insect to find the highly unpredictable host on which it realizes the greatest fitness and discuss further selective pressures that may also affect insect fitness on both hosts and remain to be tested. These results also highlight the importance of manipulative experiments with synthetic pheromones in an ecological context to help understand the evolution of the pheromone in a species' life history.

Materials and Methods

Insects

Adult *Corimelaena extensa* used for the infestation treatment in the 2010 field choice assay were collected from native *Nicotiana attenuata* and *N. obtusifolia* in or near the Lytle Ranch Preserve in southwest Utah, USA (N 37.146301, W 114.019795). Adult sex was determined by the external genitalia as described by McPherson and Sailer (1978) for *C.*

obscura. To establish a lab colony of *C. extensa*, approximately 100 adults were collected from native *N. attenuata* and *N. obtusifolia* as above, on June 14, 2009 and brought to Jena, Germany. Insects were kept in plastic boxes (9L, Lock & Lock) with a mesh lid in a Snijders growth chamber with a photoperiod of 16:8 h (light:dark, L:D), at 26°C (L) and 23°C (D), and 50% humidity, and fed on *N. attenuata* cuttings with flowers and seed capsules and Eppendorf tubes with fresh water stoppered with cotton dental rolls. Food and water was changed on a weekly basis. New adults (50-80) were brought from the same field sites and added to the colony in June 2010, July 2011, June 2012 and June 2013.

Plant germination and growth conditions

Seeds of the 30th generation of an inbred WT line of *Nicotiana attenuata* were used for glasshouse volatile collections and field assays. Seeds from the 17th generation of the same inbred *N. attenuata* line and of the 1st inbred generation of *N. obtusifolia* were used for lab feeding assays. Seeds were germinated and cultivated according to (Kruegel *et al.* 2002), no smoke treatment was used for *N. obtusifolia* seeds. Flowering plants (42-44 days after germination) were used for all lab assays. Field grown plants were cultivated in the experimental field plot at the Lytle Ranch Preserve near Santa Clara, UT, USA, as described in (Kessler *et al.* 2012).

Field choice assay on *N. attenuata* with and without conspecific feeding

On June 5, 2010, 11 size-matched pairs of flowering WT plants of the 30th generation of an inbred line of *N. attenuata* were assigned to one of two treatments: infestation by 10 adult *C. extensa* adults (7-8 males and 2-3 females, *"C. extensa* feeding") or control ("no feeding"). Insects were confined on the flower heads of the host plant with a perforated plastic bag (240x350 mm, JG Verpackungen, Schönheide, Germany) and twist tie. Flower heads of control plants were covered by perforated plastic bags to exclude feeding insects and control for effects of bags on *C. extensa* behavior. Plants were monitored daily and the number of colonizing *C. extensa* was counted during 6 consecutive days. The cumulative number of bugs at the end of the experiment on each treatment was compared using a paired Wilcoxon sign test.

Laboratory volatile collections

To detect specific cues of *C. extensa*, headspace samples were collected from the aerial parts of flowering *N. attenuata* with or without *C. extensa* feeding. In feeding treatments, 12 adult *C. extensa* (9 males and 3 females) were starved overnight and then added to flower heads of both species and allowed to feed freely within the collection chambers. Headspace collections were performed in 25-L silanized glass cylinders as described in (Späthe *et al.* 2013) on self-packed filters containing 20 mg of Porapak Q (Sigma-Aldrich). Plants were maintained in a 16:8 h (L:D) photoperiod and the headspace was sampled for 8 h during the photophase (starting 3h after onset of the photophase) and 8 h during the scotophase on three consecutive days.

Volatiles were eluted from filters and analyzed by Gas Chromatography- ion trap Mass Spectrometry (GC-ion trap MS), as previously described (Schuman *et al.* 2012). Samples were measured on a nonpolar VF-5ms column (30 m × 0.25 mm i.d. × 0.25 mm; Varian Inc., Lake Forest, CA, USA) in a Varian CP-3800 GC (Varian Inc., Palo Alto, CA, USA) equipped with a CP-8400 auto-injector in splitless mode, and analyzed on a coupled Varian Saturn 4000 ion trap mass spectrometer with the same instrument parameters described in (Schuman et al 2009; GC-EI-MS method1, supporting information). Control and *C. extensa* feeding samples were compared to search for peaks specific to the *C. extensa* feeding treatment in either day or night collections (for further details, see Supporting Information).

To compare *C. extensa* pheromone emission on both host plants, headspace samples were collected from the aerial parts of flowering *N. attenuata* and *N. obtusifolia* with *C. extensa* feeding (9 males and 3 females/ plant) in 25-L silanized glass cylinders, as described above. Volatiles were collected for 8 h during the light phase (3 h after onset of photophase) and eluted with 200 µL of DCM after spiking filters with 240 ng of β-caryophyllene as internal standard. Samples were measured by GC-EI-MS method 2 (supporting information).

Pheromone structural elucidation and *de novo* synthesis

Since the putative pheromone detected in headspace samples could not be detected in crude hexane and DCM extracts of male *C. extensa*, possibly due to other similar but more highly abundant compounds, the pheromone was isolated from pooled headspace samples of

C. extensa males actively feeding on *N. attenuata* collected and eluted in DCM as described above, and separated on an Agilent 7890A GC (Agilent) equipped with an HP5 capillary column (30 m × 0.53 mm ID with 1.5 µm film thickness, Agilent), connected to a preparative fraction collector with a cryostatic trap cooler (PFC, Gerstel), with a 2 µL splitless injection at 250°C. The oven program was 100°C for 5 min, increased at 20°C min^{-1} to 165°C, then at 2°C min^{-1} to 210°C, and held for 1 min. The carrier gas was helium with a constant flow rate (4.8 ml/min). The purified compound was used for derivatization reactions to determine the presence of a carbonyl group (microsilylation, treatment with pentafluorobenzylhydroxylamine, reduction with lithium aluminum hydride), determine the number of double bonds and carbon backbone structure (microhydrogenation)(Attygalle 1998). Finally, the exact position and geometry of the unsaturations were determined by NMR spectroscopic methods: selective TOCSY and homodecoupling experiments revealed both the double bonds to be *cis*-configured. Additional experiments including ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC identified the structure as (5Z,8Z)-tetradeca-5,8-dienal. Synthetic (5Z,8Z)-tetradeca-5,8-dienal was prepared from TBDMS protected hex-5-yn-1-ol and 1-iodooct-2-yne in a three-step reaction described in Fig. S2 and a further comparison proved the *de novo* synthesized compound to be identical with the GC-purified pheromone. Details on derivatization reactions, NMR spectroscopy, *de novo* synthesis and instrument parameters can be found in the supporting information.

Pheromone perfuming choice assay

On May 26, 2013, 13 size-matched pairs of flowering WT plants of the 30th generation of an inbred line of *N. attenuata* were assigned to one of two treatments: perfuming with (5Z,8Z)-tetradeca-5,8-dienal or solvent control. Attractiveness of (5Z,8Z)-tetradeca-5,8-dienal was tested by applying 500 µg of the *de novo* synthesized compound in 500 µL hexane on a natural rubber septum (7.9 x14 mm, Sigma-Aldrich) which was fastened to the main stem of the plants with a twist tie, approximately 15 cm below the flower heads. Control plants were perfumed with the same volume of hexane applied to rubber septa. Release of (5Z,8Z)-tetradeca-5,8-dienal from septa at 26^{o}C was of 0.95 (±0.13) µg/day and septa were exchanged for new ones every 5 days. Plants were monitored daily during 15 consecutive days and colonizing *C. extensa* were collected and stored in our field lab facilities, where their sex was determined to evaluate changes in sex ratio during the colonization and also to avoid contamination of control plants by pheromone-emitting male

colonizers. The final number of adult *C. extensa* collected on both treatment groups were compared using a Welch t test for heteroscedastic samples.

Olfactometer assays

To test whether *C. extensa* adults showed preference for one of the two tobacco hosts and if there were sex-related differences in preference, the attraction of *C. extensa* to volatiles from both host plants was analyzed using a circular olfactometer (15 cm diameter) divided into 4 quadrants in a 2-odour design, with odor containing sectors facing each other on opposite sides of the arena alternating with 2 blank sectors. Plant odors consisted of cuttings of either *N. attenuata* or *N. obtusifolia* branches containing flowers, unopened seed capsules and leaves, weighed to 1.5 g fresh mass, inside closed 150mL silanized glass bottles together with moist paper towels to maintain air humidity within the arena. Blank sectors consisted of only moist paper towels inside the bottles. Charcoal-filtered air was pushed through odor or blank 150mL glass bottles at 0.2 L/min and delivered to the arena through a hole in the center of lower surface of each sector and pulled at 1L/min from the center of the arena. The bottom half of the arena was covered with a fine mesh upon which the insects walked and contacted the odors beneath them, after being placed in the center of the arena and the arena was lit only from below using white LEDs. Assays lasted 15 min and individual insects were excluded if they did not leave the central ("no choice") zone during the first 5 min of the assay. Insects were tested individually and data from males and females were analyzed separately. All assays were recorded with a webcam and analyzed with the open source video tracking software Tracker 4.82 (Brown 2013) to evaluate time spent in each sector. Differences in time spent in each sector were analyzed as proportion of total time with a Friedman rank sum test (Vet *et al.* 1983).

Laboratory feeding assays

Insects were confined to the feeding plants using perforated plastic bags (240x800 mm, JG Verpackungen) which were placed around the entire aerial part of the plants, except for the rosette leaves, and fastened around the stem with a twist tie. The tops of the bags were supported with a ring of steel wire to keep the bag from collapsing and allow space for plant growth, and this wire was fastened to a bamboo stick to keep the bag in an upright position

during plant development. Fecundity was measured as the number of eggs and nymphs at the end of the assay. The proportion of nymphs to eggs and of fifth instar nymphs to the sum of all other juvenile stages were compared as a measure of juvenile developmental time within each density treatment. The impact of *C. extensa* feeding on both hosts was assessed by weighing a pooled sample of seeds collected at the end of the experiment and testing if feeding had any impact on seed mass compared to uninfested control plants. Control plants were also confined within perforated plastic bags as described above for the duration of the experiment. The final number of eggs and of nymphs within each treatment were analyzed a Wilcoxon sign test and the proportion of nymphs to eggs and of fifth instar nymphs to all other juvenile stages were analyzed with a Fisher exact test. Seed mass was analyzed by two-factor and one-factor ANOVAs with a Tukey HSD post hoc comparison.

Statistical analysis

All data analysis was performed using the R environment (Team 2009). When assumptions of normality and homoscedasticity were not met even after transformation, data was analyzed with non-parametric tests (Zar 1999).

Results

Corimelaena extensa males pioneer colonization of the unpredictable host *Nicotiana attenuata*

Insect volatile pheromones used in host finding and recruitment of conspecifics are frequently emitted by only one of the sexes, which acts as the pioneer in the colonization of new hosts. To assess whether this could be the case during the colonization of *N. attenuata* by *C. extensa*, we examined the sex ratio (number of males/ number of females) of the pioneer individuals and of individuals from established *C. extensa* populations collected on native *N. attenuata* plants growing around our field site in southwestern Utah. Our observations showed that males outnumber females during initial phase of *N. attenuata* colonization, but one month later, established populations in the same areas have equal numbers of males and females (Fig. 2A). Only adults were observed during this phase of host colonization. Our earliest observations of native *C. extensa* adults on *N. obtusifolia* in the field have shown that these populations have an unbiased sex ratio, as do our laboratory colonies (Fig. S1). To examine whether initial colonizers increased recruitment of conspecifics on *N. attenuata*, we

performed a choice assay and compared the recruitment of adult *C. extensa* to field grown *N. attenuata* plants infested with adult *C. extensa* in a male-biased (3:1) sex ratio to the colonization of uninfested control plants. Infestation by conspecifics significantly increased the recruitment of adult *C. extensa* colonizers compared to controls (Fig. 2B, Wilcoxon paired test, P = 0.02), indicating that the presence of feeding males increases the long-range attractiveness and/or apparency of *N. attenuata* to these herbivores.

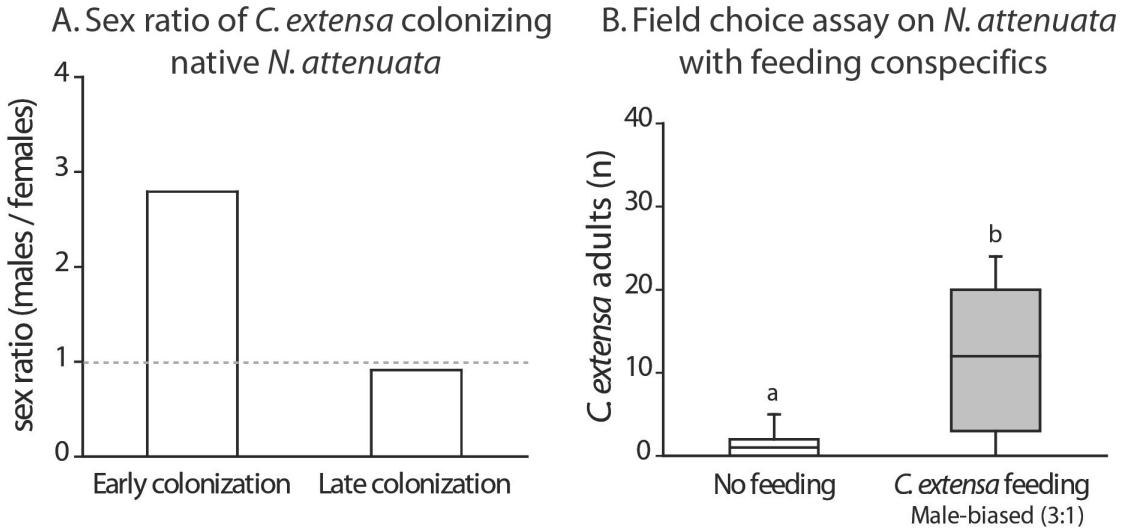

Figure 2. The initial colonization of *N. attenuata* is characterized by a male-biased population of *C. extensa* which increases the host plant attractiveness to conspecific colonists. **A**) Sex ratio (n males/ n females) of adult *C. extensa* collected on native *N. attenuata* during early colonization (June 2011, N = 91 insects) and approximately 1 month after onset of colonization (July 2011, N = 65 insects). The dotted line represents the sex ratio of stable populations (1:1). **B**) *N. attenuata* plants infested with *C. extensa* adults (3:1 male-biased sex ratio) attracted significantly more adult *C. extensa* than plants without feeding (N = 9 pairs of plants, matched for size). Feeding treatment = 9 males + 3 females/plant. Boxes comprise the 25-75 percentile, lines show median values and whiskers denote the range. Different letters represent $P<0.05$ in a paired Wilcoxon sign test.

Colonization of *N. attenuata* is mediated by an aggregation pheromone

To test the role of volatile infochemicals as long-range signals in host finding and colonization of *N. attenuata*, we compared the whole-plant headspace of *N. attenuata* infested with *C. extensa* (3:1 male-biased sex ratio) and uninfested control plants. An unbiased GC-MS analysis revealed one unknown compound in the volatile profile of *C. extensa* infested *N. attenuata* (Fig. 3A) which was lacking in uninfested plants. The unknown volatile was present in samples collected during the photophase, but only traces were detected

during the scotophase (data not shown). We purified this compound from headspace samples using GC-PFC and through derivatization reactions and NMR measurements comparing the purified compound to a *de novo* synthesized standard, we identified the compound as (5Z, 8Z)-tetradeca-5,8-dienal (see Supporting Information and Figs. S2-7 for details on synthesis and structural elucidation). Further experiments showed that this volatile is emitted by feeding male adult *C. extensa*, and continues to be emitted 90 min after removal from plants, but is absent from the headspace of feeding females (Fig. S8) and of nymphs (data not shown), suggesting that this compound is a male-produced pheromone which mediates the colonization of this host plant by *C. extensa*.

To confirm the role of (5Z, 8Z)-tetradeca-5,8-dienal as the long-range attraction cue in the colonization of *N. attenuata* we performed a perfuming experiment on field-grown *N. attenuata* with the *de novo* synthesized standard. *N. attenuata* plants perfumed with synthetic (5Z, 8Z)-tetradeca-5,8-dienal diluted in hexane attracted significantly more native *C. extensa* than control plants perfumed with pure hexane (Fig. 3B, Wilcoxon paired test, $p = 0.01$). Adults of both sexes responded to synthetic (5Z, 8Z)-tetradeca-5,8-dienal and the sex ratio of recruited bugs followed the same pattern observed in natural colonization of this host plant, with an initial male bias at the start of the experiment (sex ratio on day 1 = 2.8) and a linear decrease in sex ratio after 15 days (sex ratio on day 15 = 0.9; Fig. 3C, intercept = 3.307, slope = -0.143, adjusted $R^2 = 0.64$, $p < 0.001$), supporting our hypothesis that this compound acts as an aggregation pheromone of *C. extensa*. It's important to note that since insects were removed after daily checks, the final sex ratio of adults collected in this experiment was still slightly male-biased. This is likely due to the interruption of the experiment after only two weeks, while in natural populations it can take a month for a population to become established in a 1:1 ratio on *N. attenuata* (see Fig 2A).

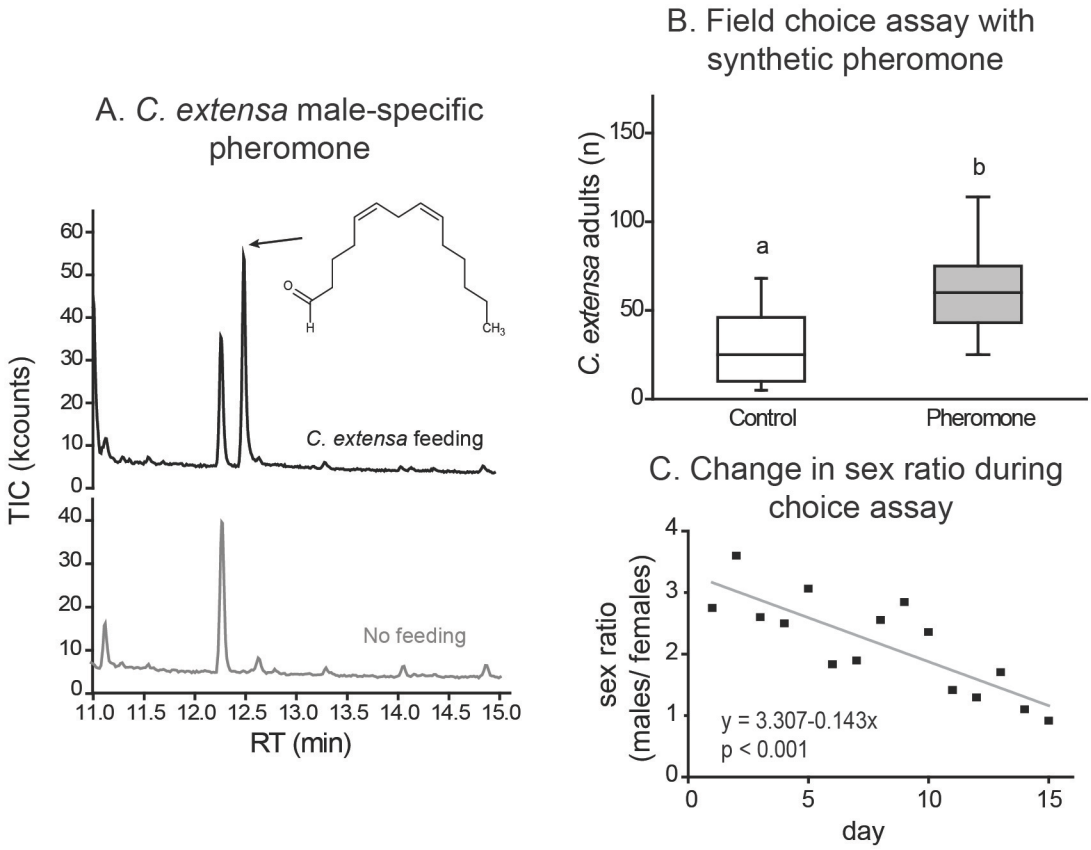

Figure 3. Male *C. extensa* feeding on *N. attenuata* emit an aggregation pheromone which attracts conspecific adults of both sexes. **A**) The headspace of *N. attenuata* plants infested with adult *C. extensa* (top chromatogram) differed from non-infested control plants (bottom chromatogram) by one major compound, the putative pheromone, (5Z, 8Z)-tetradeca-5,8-dienal (structure shown above corresponding peak). For details on volatile analysis see text. **B**) Significantly higher numbers of adult *C. extensa* were collected on *N. attenuata* plants perfumed with 1mg synthetic (5Z, 8Z)-tetradeca-5,8-dienal dissolved in Hexane compared to solvent controls (N =13 plant pairs, matched for size). Boxes comprise the 25-75 percentile, lines show median values and whiskers denote the range. Different letters represent $P<0.05$ in a paired Wilcoxon sign test. **C**) The sex ratio of colonizing adults collected in the field choice assay transitioned from initial male-bias to an unbiased ratio over the 2 weeks measured, similar to native populations.

Plasticity in the emission of the aggregation pheromone reflects adult host plant preference

Due to the observed differences in colonization behavior of *C. extensa* on both tobacco hosts, we tested whether these differences were caused by a difference in preference for either *N. attenuata* or *N. obtusifolia,* and if this was reflected in the emission of the aggregation pheromone to recruit conspecifics. To assess differences in host plant preference, we performed olfactometer two-choice assays using the headspace odors of uninfested *N.*

attenuata and *N. obtusifolia* plants. Fig. 4A shows that both males and females spent longer amounts of time in the arena quadrant containing *N. attenuata* odors when offered a choice between both hosts (proportion time spent in sector, Friedman Rank Sum Test, males: χ^2 = 24.37; df =1; p < 0.001; females: χ^2 = 7.54; df = 1; p = 0.006). Results from a separate choice assay where excised flowers and green seed capsules of both hosts were offered to adult *C. extensa* inside a 10x5x3 cm plastic culture box showed that apart from attraction to host volatiles, *C. extensa* also prefer to feed on *N. attenuata* (Fig. S9). To investigate differences in the emission of the aggregation pheromone on both hosts, we measured the emission of (5Z, 8Z)-tetradeca-5,8-dienal by *C. extensa* after 48h of feeding on either *N. attenuata* or *N. obtusifolia*. Headspace analysis of *N. attenuata* and *N. obtusifolia* plants infested with adult *C. extensa* in the glasshouse revealed that *C. extensa* emits on average 40 times higher amounts of the aggregation pheromone after 48h feeding on *N. attenuata* when compared to bugs feeding on *N. obtusifolia* (Fig 4B, Welch t test, t = 4.998, P = 0.004). These results show that there is a host-specific plasticity in the emission of the aggregation pheromone with higher levels of pheromone emission on the preferred host plant, *N. attenuata*.

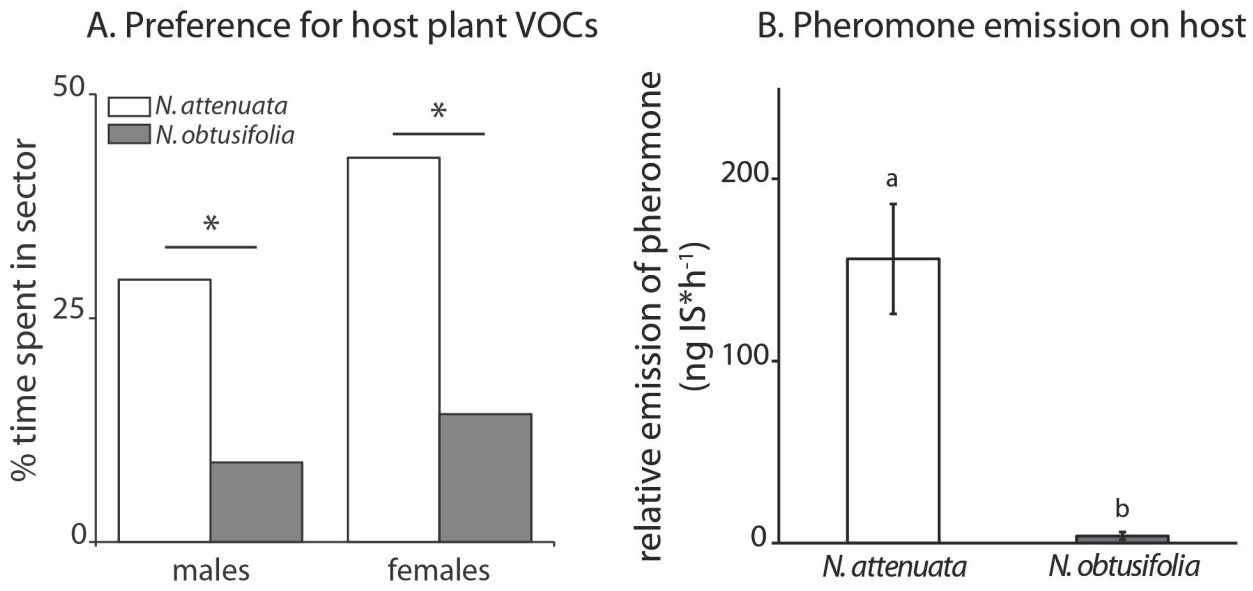

Figure 4. The emission of (5Z, 8Z)-tetradeca-5,8-dienal on both host plants reflects adult preference for the unpredictable host. **A**) Both male and female adult *C. extensa* preferred uninfested *N. attenuata* volatiles over uninfested *N. obtusifolia* volatiles in a 4-sector choice arena with odors in 2 sectors (see text for details). Asterisks represent significant differences in a Friedman Rank Sum test comparing percentage of total time spent in the two sectors with host plant odors. **B**) Emission of the aggregation pheromone was significantly higher by adult *C. extensa* feeding on *N. attenuata* compared to those feeding on *N. obtusifolia* (2-Factor ANOVA, 9 males: 3 females/plant; N = 6 cross-species pairs, matched for phenological stage). For details of volatile collection and analysis are see text. Bars represent means ± standard error.

Communal feeding on *N. attenuata* significantly increases insect fitness and decreases host fitness

To test whether there are differences in *C. extensa* insect performance on both host plants which could be related to the observed differences in preference and pheromone emission, we measured the fecundity of adults feeding for 21 days on *N. attenuata* and *N. obtusifolia* during an on-plant feeding assay. To further investigate if these insects derive a fitness benefit from communal feeding, we manipulated initial adult density to establish a low density (LD, 2 males and 2 females per plant) and a high density (HD, 10 males and 10 females per plant) feeding treatment on both hosts. Fecundity was measured as the final number of nymphs and eggs in each experimental group and compared between hosts. Since nymphs and eggs are not independent variables, the final proportion of nymphs to eggs at each density level was used as a measure of reproductive rate within host plant and the proportion of 5^{th} instar nymphs (relative to the sum of nymphs from the 1^{st} to 4^{th} instars) was used as an estimate of juvenile development time.

Fig. 5A shows that *C. extensa* has large fitness benefits from using *N. attenuata* as a host plant. Host differences in the LD treatment were caused by significantly higher number of eggs laid by *N. attenuata* fed bugs, but not of nymphs (Wilcoxon sign test, eggs: $W = 45$, $P = 0.006$; nymphs: $W = 28$, $P = 0.699$). In contrast, there were no significant differences in the number of eggs laid on both hosts in the HD treatment, but there was a significantly higher number of nymphs among the offspring of *N. attenuata* fed bugs (Wilcoxon sign test, eggs: $W = 27.5$, $P = 0.749$; nymphs: $W = 47$, $P = 0.002$) compared to *N. obtusifolia*, and this difference led to a higher nymph-to-egg ratio in the *N. attenuata* fed HD treatment (Fisher exact test $P < 0.001$). Additionally, there was a higher proportion of 5^{th} instar nymphs among *C. extensa* feeding on *N. attenuata* in the HD treatment compared to the same density group on *N. obtusifolia* (Fisher exact test, $P < 0.001$), but no differences were observed between hosts in the LD treatment (Fisher exact test, $P = 1$). Together these data suggest that the reproductive rate of *C. extensa* is higher and the juvenile development time is shorter when feeding on *N. attenuata*.

In the within-host plant comparisons, HD feeding on *N. attenuata* led to an increased proportion of nymphs to eggs compared to LD (Fisher exact test, $P < 0.001$), due to an increase in number of nymphs, but not eggs, in the HD group (Wilcoxon eggs: $W = 24.5$, $P = 1$; nymphs: $W = 49$, $P = 0.002$); however there were no differences in the proportion of 5^{th}

instar nymphs between LD and HD groups. This indicates that although *C. extensa* has an increased reproductive rate when feeding in higher densities on *N. attenuata,* this is not caused by a faster juvenile development rate, but likely an earlier onset of reproduction in the HD group. On *N. obtusifolia*, both the proportion of nymphs to eggs (Fisher exact test, $P < 0.001$) and the proportion of 5^{th} instar nymphs (Fisher exact test, $P < 0.001$) were significantly different between LD and HD groups. This was mainly caused by a sharp decrease in eggs laid by the LD feeding group and consequently higher proportion of nymphs in this group at the end of the experiment, which suggests that, in addition to the slower juvenile development, adult fitness is also strongly limited on this host plant. Adult mortality was not significantly different between hosts, but there was a significant density effect with higher mortality observed on bugs feeding in HD group compared to the LD on *N. obtusifolia* (arc-sine transformed data, two-factor ANOVA, Treatment: $F = 6.23$, d.f.=1, $P = 0.020$; Tukey HSD: $P = 0.034$).

Both host plants also varied in regard to their responses to the different levels of *C. extensa* feeding (Fig. 5B). As shown previously (Anssour *et al.* 2009), seed mass of control *N. obtusifolia* plants is significantly lower than that of control *N. attenuata* (Fig. 5B, two-factor ANOVA, Host plant: $F = 405.40$, d.f. = 1, $P < 0.001$) and therefore within-genotype differences were analyzed separately by one-factor ANOVA. The seed mass of *N. attenuata* was significantly lower in the HD treatment compared to uninfested control plants (one-factor ANOVA, $F = 14.69$, d.f. = 2, $P < 0.001$; Tukey HSD, $P < 0.001$) but not in the LD treatment (Tukey HSD, $P = 0.846$). Feeding by *C. extensa* on *N. obtusifolia* did not have any impact on seed mass compared to uninfested controls (Fig. 5B, *N. obtusifolia*: one-factor ANOVA, $F = 0.37$, d.f. = 2, $P = 0.697$). These patterns were reflected in the percentage of viable seeds in both hosts (arc-sine transformed data, two-way ANOVA, treatment: $F = 11.5$, $P = 0.0002$; treatment x host: $F = 9.5$, $P = 0.0006$). Seed viability in *N. attenuata* was significantly lower in the HD treatment (66.6% ±3.6) but not in the LD (93.4% ±3.2) group compared to control (98.3% ±1.1; Tukey HSD, $P < 0.001$ and $P = 0.816$, respectively). There were no differences in viability between feeding treatments (LD: 92.4% ±3.4; HD: 84.3% ±5.1) and control in *N. obtusifolia* (83.0% ±3.7). Viability values are average ±SE (N = 5-7). These results suggest that *N. attenuata* is more susceptible to feeding damage by *C. extensa*, at least at higher infestation densities.

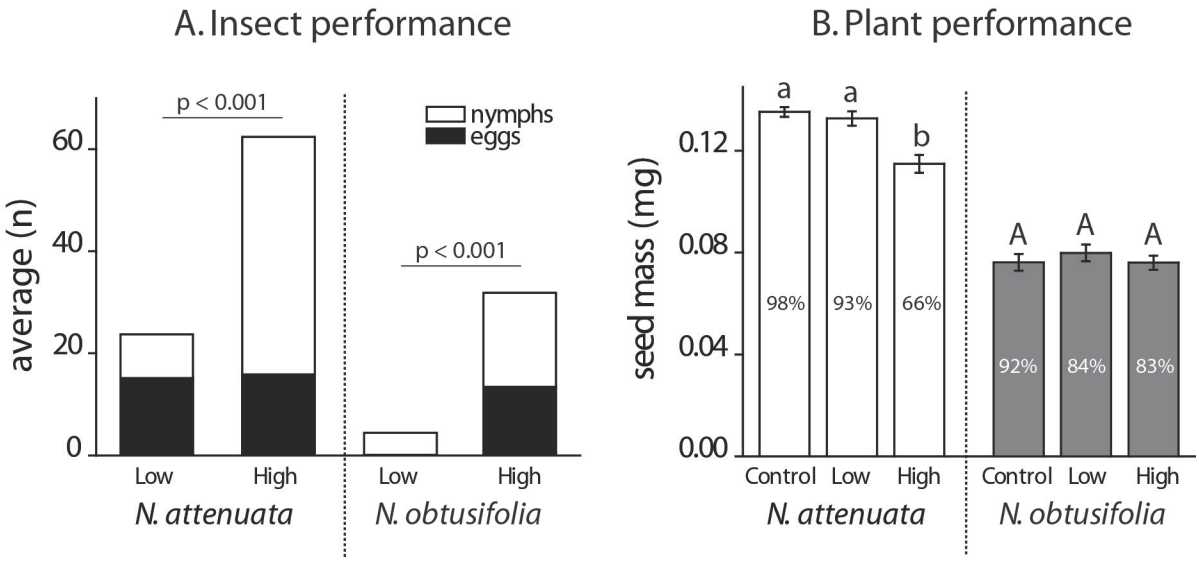

Figure 5. *C. extensa* realized higher fecundity when feeding on *N. attenuata* compared to *N. obtusifolia*, and communal feeding has a negative impact on *N. attenuata* seed mass and viability. **A**) Adults feeding on *N. attenuata* produced more nymphs and eggs than those feeding on *N. obtusifolia* after 21 days. Two levels of initial adult density treatments were used on both host plants, Low: 4 adults (2 males + 2 females) and High: 20 adults (10 males + 10 females) per plant (N =7 plants/ density). P values represent the comparison of the proportion of nymphs and eggs within each genotype compared using a Fisher Exact Test for count data. **B**) High density communal *C. extensa* feeding has negative impacts on individual seed mass of *N. attenuata* but not *N. obtusifolia*. Control plants were not infested with *C. extensa*. Different letters represent significant differences in seed mass (Two-Factor ANOVA, Tukey HSD Post-Hoc). Percentage viable seeds are written inside columns (see text for details).

Discussion

In this study we show that male adults of *C. extensa* act as pioneers during the colonization of the unpredictable fire-chasing annual host plant *N. attenuata*, but not on the closely related perennial *N. obtusifolia* although they also use this plant as host (Fig. 2). Interestingly, the lack of male-bias observed when *C. extensa* feeds on *N. obtusifolia* (Fig. S1) is also absent from a report of this species feeding on another perennial tobacco, *N. glauca*, in California (Lung & Goeden 1982). In their study, Lung & Goeden report that *C. extensa* can be found throughout the year feeding on *N. glauca* in non-desert areas of southern California, and this suggests that in the absence of dispersal and colonization of a new host, *C. extensa* populations normally have 1:1 sex ratio, similar to that of established populations feeding on *N. attenuata* and of our non-diapausing lab colonies (Fig. 2 and S1). Although it is reported that *Corimelaena* spp. overwinter in the leaf litter (McPherson 1982), it is possible that in the absence of leaf litter such as in our study area *C. extensa* may overwinter in rocky outcrops as reported for *Lygaeus equestris* (Solbreck & Kugelberg 1972).

Such overwintering sites would likely be close to *N. obtusifolia* populations which have a longer flowering cycle than *N. attenuata* (Fig. 1) and persist over many seasons, and it is likely that this plant serves as an alternate host in early spring and perhaps in late autumn in the absence of flowering *N. attenuata* populations.

In a manipulative field assay, we also show that a newly identified aggregation pheromone, (5Z, 8Z)-tetradeca-5,8-dienal, emitted by male adults mediates the aggregation of *C. extensa* on *N. attenuata* (Fig. 3). Furthermore, we show that the lower emission of this pheromone by *C. extensa* feeding on *N. obtusifolia* is consistent with the smaller insect population densities observed on this host and with lab choice assays which show a preference of *C. extensa* for *N. attenuata* over *N. obtusifolia* (Fig. 4, Fig. S9). We propose three non-mutually exclusive hypotheses to explain the observed host plant-mediated plasticity in pheromone emission and colonization behavior of *C. extensa* on both tobacco hosts: (A) the plasticity in pheromone emission provides a reliable signal of host quality and/or identity; (B) differences in pheromone biosynthesis are caused by differences in abundance of a plant-derived chemical precursor between hosts; and (C) predation and/or parasitism pressure is higher on *N. obtusifolia*, leading to a selective pressure against pheromone emission on this host.

Two of the pre-requisites for hypothesis (A) are a consistent difference in pheromone emission which would constitute a reliable signal and an associated fitness benefit of feeding on *N. attenuata*. Our data shows that the pheromone emission of *C. extensa* on both hosts shows a striking quantitative difference in emission (Fig. 4) indicating that it could serve as a reliable signal of the host plant. Similarly, a study of host plant colonization by milkweed bugs suggested that higher pheromone content in male *Lygaeus kalmii* to facilitate aggregation of more widely scattered individuals which was caused by its broader host range compared to the more specialized *Oncopeltus fasciatus* (Aldrich *et al.* 1999). Plasticity in pheromone emission can be qualitative (changes in the ratio of compounds of a blend) or quantitative (total amounts). While intraspecific plasticity in ratios of pheromone components has been implicated as a pre-zygotic barrier against hybridization in sympatric *Heliothis* spp. moths (Groot *et al.* 2010), it is more commonly reported in formation of geographical or host-races which may lead to speciation, and a number of studies suggest that intraspecific quantitative variation in pheromone emission is more common than variation in the ratio of compounds in the blend (Roelofs *et al.* 1987; Schlyter & Birgersson 1989; Johansson &

Jones 2007; Groot *et al.* 2008). Although it is known that host plants influence pheromone emission through feeding-stimulated increases in production and release of these infochemicals compared to starved insects (reviewed in Landolt & Phillips 1997), few studies have focused on whether host plants have a direct effect on shaping the plasticity in pheromone emission and quantitative individual variation in pheromone emission is viewed mostly as a consequence of the costs of pheromone production and physiological state of the individual.

One example of a host plant shaped plasticity in pheromone emission is the pheromone blend of the bark beetle *Ips pini* which feeds on three related pines (*Pinus* spp.) and contains a *de novo* synthesized pheromone (ipsdienol) and plant-derived monoterpenes. When beetles fed on the white pine *Pinus strobus* the blend contained a higher pheromone to monoterpene ratio compared to beetles feeding on *P. banksiana* and *P. resinosa* (Aukema *et al.* 2010). This change is consistent with increased attraction of conspecifics observed on *P. strobus* in the study area, and is similar to our observations of *C. extensa* on *N. attenuata*. However, success rates of attacks by *I. pini* on *P. strobus* are lower than on the other two hosts, possibly due to increased resin defenses in this tree. In contrast, our data from the feeding assay on both hosts show that adult realized fecundity (number of offspring produced) is higher and juvenile development time is shorter on *N. attenuata* than on *N. obtusifolia* (Fig. 5A), suggesting the possibility that the higher pheromone emission on *N. attenuata* serves as an indicator of a high quality host, in support of hypothesis (A). The higher realized fitness on *N. attenuata* together with the increase in nymph/egg ratio indicates an earlier onset of reproduction in *C. extensa* feeding in higher densities compared to the low density feeding group on *N. attenuata* and also suggests a benefit of forming an aggregation on this host. Furthermore, the observed decrease in host plant seed weight and viability after high density feeding by *C. extensa* in our experiment (Fig. 5B) suggest that benefits of communal feeding could include better resource use or possibly overcoming of host plant defenses, but further studies are needed to dissect the mechanism through which communal feeding increases *C. extensa* fitness on this host.

While we cannot fully exclude hypothesis (B) without further characterization of the biosynthesis of (5Z, 8Z)-tetradeca-5,8-dienal using labeled precursors, other studies have shown that the most fatty-acid derived pheromones from different insect taxa such as moths, beetles, cockroaches and also true bugs, are *de novo* synthesized by the insects (Tillman *et al.*

1999; Millar 2005); and fatty acids such as myristic and linoleic acids have been reported to be biosynthesized in aphids (Ryan *et al.* 1982; De Renobales *et al.* 1986). Therefore we speculate that host plant may not strongly influence precursors available for pheromone biosynthesis in *C. extensa*, but this hypothesis requires further testing.

So far we know little of the natural enemies of *C. extensa*, so hypothesis (C) also requires further field bioassays to assess the relative importance of predators on pheromone emission and affect host preference by *C. extensa*. While it is known that natural enemies can affect host plant choice by herbivores and can use herbivore pheromones to locate their prey (Aldrich *et al.* 1999; Dicke 2000; Allmann *et al.* 2013; Martinez *et al.* 2013), it has also been shown that in some plant bugs (Miridae) chemicals that act as pheromones can also act as defensive compounds or have a repellent effect, in a dose-dependent manner (Millar 2005). In short, further studies are necessary to see if natural enemies exert a positive or negative selective pressure on the observed pheromone emission and host choice by *C. extensa*.

The factors outlined in hypotheses (B) and (C) can certainly contribute to the definition of host quality in hypothesis (A), but a common thread in all three explanations proposed is the influence of host plant chemistry, both in terms of nutrient and defensive chemical content ("host plant quality", Fig. 1), which is likely to mediate most, if not all, of these aspects. Apart from constitutive and herbivore induced volatile compounds which can affect host finding and preference, the two tobacco species studied here are known to differ in defensive metabolite content. *N. obtusifolia* is known to have higher levels of nornicotine and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) while *N. attenuata* has higher levels of nicotine and trypsin proteinase inhibitors (TPIs), which affect the associated insect community, including the specialist tobacco hornworm, *M. sexta*, which is less frequently found on *N. obtusifolia* (Baldwin & Ohnmeiss 1993; Wu *et al.* 2006; Jassbi *et al.* 2010).We would therefore also expect to find differences in associated communities of natural enemies of the herbivores on both plants, which would affect hypothesis (C). Additionally, plant chemicals don't only provide precursors for pheromones (hypothesis B) but may also be sequestered by herbivores for their own defense against natural enemies (e.g. Aldrich *et al.* 1997), although it is also known that seeds of *N. attenuata* contain little nicotine and no TPIs (Baldwin 1999; Van Dam *et al.* 2001). Finally, the interaction of nutrients and defensive chemicals can have direct impact on herbivore growth, development and fecundity (Schwachtje & Baldwin 2008; Orians *et al.* 2011), and ongoing research seeks to assess

which differences in defensive and primary metabolites of *N. attenuata* and *N. obtusifolia* can explain the observed differences in realized fitness of *C. extensa*.

In conclusion, through application of chemical structure analysis and use of a synthetic pheromone to address ecological hypotheses in lab and field settings, we show that the seed-feeding true bug *C. extensa* shows predictable quantitative plasticity in the release of its aggregation pheromone on two different host plants which differ in their spatio-temporal predictability due to differences in life history strategies. We propose that this pheromone emission plasticity serves as a reliable and specific cue to coordinate the colonization of the unpredictable host plant on which *C. extensa* realizes higher fitness, effectively using the pheromone to make a non-apparent host plant apparent to conspecifics and thereby reducing its spatial unpredictability. We also show evidence that host plant species shapes this plasticity in pheromone emission and future studies will address the mechanisms through which both host plants influence the production and release of this infochemical.

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Supporting Information: supporting methods

1) Determination of male-specific volatiles

To identify if males or females emitted the putative pheromone, we collected volatiles from males and females separately feeding on plant cuttings of *N. attenuata* in a closed-loop collection system described in (Beran *et al.* 2011) using 250mL silanized glass Erlenmeyer flasks (N= 5 insects/plant, 6 replicates per sex). Insects were allowed to feed for 24h and then volatiles were collected for 30 min during the photophase. Immediately after collection, insects were removed from plant material and transferred to new flasks and volatiles were collected over 30 min periods from 0 to 90 min after transferal. Only the data from 60-90 min period is shown as "After Removal" (Fig. S8), the putative pheromone was only observed in the headspace of males both with and without host plant material. Porapak filters were eluted and GC-MS analysis was performed with "*GC-EI-MS method 2*" as described below. A separate experiment measuring release of the putative pheromone from flower heads on which males had been feeding immediately after insect removal only showed traces of the pheromone, in much lower abundance than in the headspace of isolated insects (data not shown).

2) GC-MS analysis

After volatile collection filters were spiked with 320 ng of Tetralin as internal standard immediately before elution and then eluted with 250 µL dichloromethane (DCM) and pushed through filters with a gentle stream of N_2 into a 2 mL glass vial (GC vial) containing a 250 µL insert. Samples were measured on a nonpolar VF-5ms column (30 m × 0.25 mm i.d. × 0.25 mm; Varian Inc., Lake Forest, CA, USA) in a Varian CP-3800 GC (Varian Inc., Palo Alto, CA, USA) equipped with a CP-8400 auto-injector operated in splitless mode, and analyzed on a coupled Varian Saturn 4000 ion trap mass spectrometer.

GC-EI-MS method 1: helium carrier gas, 1 µl of a 30s splitless injection at 250°C, initial temperature 40°C for 5 min, increased at 5°C min^{-1} to 185°C, and at 30°C min^{-1} to 300°C, 10-min hold; Electron impact (EI) ionization mode, transfer line at 230°C, trap temperature 180°C, scan range from 40 to 399 *m*/*z* at 1 spectra s^{-1}. Individual volatile

compound peaks were quantified by peak areas of two specific and abundant ion traces per compound using MS Work Station Data Analysis software (Varian) and normalized by the 104+132 ion trace peak area of the IS (tetralin) in each sample. The identification of compounds was conducted by GC retention time compared to pure standards and mass spectra compared to standards and mass spectra databases, Wiley version 6 (Wiley) and NIST (National Institute of Standards and Technology) spectral libraries and control and *C. extensa* feeding samples were compared to search for peaks specific to the *C. extensa* feeding treatment in either day or night collections.

GC-EI-MS method 2: helium carrier gas, 1 µl of 30s splitless injection at 250°C, initial temperature 100°C for 5 min, increased at 12°C min^{-1} to 160°C, and at 2°C min^{-1} to 180°C, and at 20°C min^{-1} to 300°C, 0.75-min hold; MS, EI ionization mode, transfer line at 230°C, trap temperature 180°C, scan range from 40 to 249 *m*/*z* at 3 spectra s^{-1}. The pheromone peak was quantified by peak area of 67+79+91 ion traces using MS Work Station Data Analysis software (Varian) and normalized by the 91+133 ion trace peak area of the IS (β-caryophyllene) in each sample. The assignment of the pheromone was conducted by use of the Kovats Index compared to a C8-C20 alkane mix and confirmed by GC retention time and mass spectra comparison to the *de novo* synthesized standard (see "Pheromone structural elucidation and *de novo* synthesis" section below).

GC-MeOH-CI method: GC settings were the same as in *GC-EI-MS method 2*, MS, CI Auto ionization mode, reaction time 40 ms, transfer line at 230°C, trap temperature 180°C, scan range from 70 to 299 *m*/*z*.

3) Derivatization reactions with the isolated pheromone

Microsilylation: 500 µL of N-methyl-(trimethylsilyl)-trifluoroacetamide (MSTFA) was added to an aliquot (~50 ng of purified compound in 50µL hexane) in a GC vial, vortexed and left at room temperature (RT) for 4 h. A 1µL aliquot was analyzed on GC sector field MS in EI mode using the same column oven conditions as GC-EI-MS method2 above and compared to a non-derivatized sample. The derivatization showed that the structure had 1 O atom, which agreed with high-resolution measurements which showed that the accurate mass of the putative pheromone was 208.1817 and identified the sum formula as $C_{14}H_{24}O$.

Derivatization with Pentafluorobenzylhydroxylamine (PFBHA): PFBHA (98%, Sigma-Aldrich) was added in excess to approximately 50 ng of the purified compound in 100 µL methanol (MeOH) in a GC vial, vortexed and left at RT for 2 h. After which 50 µL of H_2O was added to the sample and it was extracted with 100 µL hexane according to Becker *et al* (1976). The non-polar phase was separated and concentrated under a gentle stream of N_2 to approximately 50µL and a 1µL aliquot was analyzed on a Varian CP-3800 GC coupled with a Varian Saturn 4000 ion trap MS, using the *GC-EI-MS method 2* described above with a 40-499 *m/z* scan range. Based on the expected two peaks containing the diagnostic fragment *m/z* 181, we assigned a carboxyl group to the putative pheromone.

*Reduction of carbonyl group to a hydroxyl group:*10% excess of lithium aluminum hydride ($LiAlH_4$) in 1mL diethyl ether was added to a 50ng aliquot of sample suspended in 200 µL diethyl ether and stirred for 4 h at RT. The reaction was quenched by adding H_2O on ice and the precipitate was dissolved by adding 10% sulfuric acid (H_2SO_4) (Becker *et al.* 1976). The non-polar phase was separated and concentrated under a gentle stream of N_2 and re-suspended in approximately 50µL DCM and a 10µL aliquot was analyzed on a Varian CP-3800 GC equipped with a nonpolar VF-5ms column (30 m × 0.25 mm i.d. × 0.25 mm; Varian Inc., Lake Forest, CA, USA), coupled with a Saturn 3800 ion trap MS in Chemical Ionization mode using MeOH as the reagent gas (MeOH-CI), using the *GC-MeOH-CI method* described above. The product showed an increase in the [M+H] ion from *m/z* 209 to *m/z* 211, indicating a successful reduction of the carbonyl group.

Microhydrogenation: A small aliquot (100 µL) of the sample in MeOH was placed in a GC vial together with ~0.5mg of Rd on alumina (Sigma-Aldrich). A balloon with H_2 was attached to the vial and the sample was stirred at room temperature overnight (ca. 16 h). The sample was filtered together with MeOH and H_2O on silanized glass wool to yield ~500µL total volume, which was then extracted with 600µL hexane (modified from Attygalle 1998). The non-polar phase was separated and concentrated under a gentle stream of N_2 to approximately 50µL and a 10µL aliquot was analyzed on a Varian CP3800GC- coupled with a Varian Saturn 3800 ion trap MS using the *GC-MeOH-CI method* described above. The retention time and mass spectra of the derivatization product was compared to a tetradecanal standard (TCI Europe), to confirm the carbon backbone structure and number of C-C double bonds. The [M+H] showed and increase from *m/z* 209 to *m/z* 213, indicating the presence of 2 C-C double bonds. Comparison of the hydrogenation product to the tetradecanal showed the same major fragments standard suggesting the pheromone has a similar aliphatic aldehyde backbone.

Methylthiolation of C-C double bonds: Approximately 50 ng of sample in 20 µL hexane were derivatized with 2 µL dimethyl disulfide (Sigma-Aldrich) together with 20 µL 5% Iodine in ether , vortexed and left at RT for 24h as previously described (Attygalle 1998). After which, the iodine was decolorized with a few drops of 5% aqueous sodium thiosulphate, the sample was extracted with hexane and the non-polar phase was separated and concentrated under a gentle N_2 stream and 1 µL was analyzed on a GC-sector field EI-MS. Due to partial reaction, only the double bond at position 8 could be confirmed.

Analysis of C-C double bonds by acetonitrile (ACN)-Chemical Ionization: Aliquots of the putative pheromone and of the pheromone after reduction with $LiAlH_4$ were analyzed by GC-ACN-MS and GC-ACN-MS-MS on a Varian 450 GC (Varian Inc., Palo Alto, CA, USA) with a coupled Varian 240 ion trap MS, using a nonpolar VF-5ms column (30 m × 0.25 mm i.d. × 0.25 mm; Varian Inc., Lake Forest, CA, USA): GC, helium carrier gas 1ml min^{-1}, 1 µl of 30s splitless injection at 250°C, initial temperature 100°C for 5 min, increased at 5°C min^{-1} to 300°C and held for 5 min; MS, ACN-CI ionization mode, ion source temperature of 90°C, transfer line at 40°C, trap temperature 90°C, scan range from 65 to 500 *m*/*z*. CI-MS-MS experiments were conducted on the $[M+54]^+$ ion, as described in (Kroiss *et al.* 2011). Due to large water-loss which increased the complexity of the resulting fragments in MS-MS mode, only one of the double bonds could be tentatively assigned to position 8 and complete assignment was done by NMR.

4) Synthesis of (5Z,8Z)-tetradeca-5,8-dienal

The synthesis of (5*Z*,8*Z*)-tetradeca-5,8-dienal (**5**) was performed in a three step reaction summarized in Fig. S2. Starting with TBDMS protected hex-5-yn-1-ol (**2**) and 1-iodooct-2-yne (**1**), derived from 1-bromooct-2-yne, the diyne was prepared (**3**) applying Copper catalysis (Lapitskaya *et al.* 1993). The reduction to diene (**4**) was carried out under ambient pressure with H_2 and P2-nickel catalysis (Boland & Jaenicke 1981) in 100% z-stereo-selectivity. After removing the TBDMS-protecting group, the free alcohol was oxidized with DMP under mild conditions according to (Meyer & Schreiber 1994) in CH_2Cl_2. The reaction was observed by GC-MS until its completion, followed by filtration through a short Florisil-column. The filtrated reaction mixture was then evaporated to dryness and the residue was resolved and filtrated in pentane to give pure (5*Z*,8*Z*)-tetradeca-5,8-dienal (**5**) as a colorless liquid with strong smell. ^1H, 400MHz, $CDCl_3$, ppm; 9.73, s, 1H; 5.27-5.39, m, 4H; 2.74, t, 2H; 2.42, t, 2H; 2.07-2.11, q, 2H; 1.99-2.04, q, 2H; 1.66-1.71, p, 2H; 1.24-1.36, m,

6H; 0.86, t, 3H; ^{13}C, 400MHz, $CDCl_3$, ppm; 202.8; 130.7; 129.66; 128.71; 127.68; 43.49; 31.71; 29.51; 27.42; 26.66; 25.81; 22.78; 22.15; 14.28; GC-MS, DB-5, He; 79, 100 %; 80, 62 %; 81, 52 %; 84, 41 %; 91, 34 %; 93, 26 %; 95, 34 %; 97, 20%, 98, 24 %; 112, 20 %; 119, 8 %; 123, 10 %; 124, 6 %; 133, 8 %; 135, 2 %; 137, 18 %; 147, 2 %; 151, 8 %; 152, 4 %; 164, 4 %; 165, 12 %; 179, 2 %; 190, 2 %; 193, 2 %; 208, 2 %.

5) Structure elucidation of the *Corimelaena extensa* aggregation pheromone (5Z,8Z)-tetradeca-5,8-dienal by NMR

NMR experiments were conducted on a 500 MHz Bruker Avance spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a 5 mm TCI cryoprobe with z-gradient operating at 500.13 MHz for ^{1}H and 125.758 MHz for ^{13}C. Prior to measurements the samples were dissolved in 30 µL $CDCl_3$ and transferred into 1 mm glass capillaries which were subsequently fused to prevent evaporation during the measurement. This capillary was placed into a 1.7 mm capillary filled with deuterated benzene as external lock standard. All measurements were conducted at 300K using Bruker standard pulse programs.

For the determination of the double bond geometries, a selective TOCSY experiment was performed irradiating the resonance at δ_H 5.35 with a Gaussian pulse of 100 Hz width and 30 ms mixing time. As a result, neighboring resonances at δ_H 2.90, 2.26 and 2.19 appeared (Fig. S3). Homodecoupling experiments saturating the resonances at δ_H 2.90 and 2.26 allowed for the extraction of coupling constants from the simplified double bond multiplet, which were determined to be 10.8 and 11.1 Hz, respectively. Both the unsaturations were thus *cis*-configured. The entire structure was then characterized by long-term measurements of the ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC spectra which allowed for the complete assignment of all positions in the molecule (Figs. S4-6). A further comparison proved the *de novo* synthesized compound to be identical with the GC-purified pheromone (Fig. S7).

6) Choice assay with excised flowers and seed capsules

To test if *C. extensa* post-alightment host selection was correlated to host finding via volatiles, we performed a choice assay in which equal numbers of excised flowers and unripe seed capsules were offered to *C. extensa* adults in a 10x5x3 cm plastic culture box (6

adults/box, n = 6). Two newly opened flowers and two unripe seed capsules of each host were placed in the box so that samples from each host were approx. 8 cm apart on either side of the box. Bugs were starved overnight and placed in the center of the box and the number of bugs feeding on each host was recorded after 1 and 24h, but since the timepoints did not differ significantly, only the data for 24h is shown. Males and females were tested separately, and both sexes showed a preference for feeding on *N. attenuata* compared to *N. obtusifolia*. The total number of bugs on each host was compared by a paired Wilcoxon test.

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Supporting Figures

Fig S1. Sex ratio (n males/ n females) of *C. extensa* adults collected on native *N. obtusifolia* measured in two different years (May 2010, April 2013) and on *C. extensa* collected on a native *N. attenuata* population in 2010 and of one of our non-diapausing lab colonies fed on *N. attenuata* (measured in October 2011).Note that in 2010 *C. extensa* were collected on *N. attenuata* 1 month later than on *N. obtusifolia* because in May there were no *C. extensa* feeding on these *N. attenuata* populations. The population observed on *N. obtusifolia* in April 2013 is the earliest seasonal colonization observed in our field site in the past 5 years.

Fig. S2. A) Schematic representation of the synthesis of (5*Z*,8*Z*)-tetradeca-5,8-dienal. Starting with TBDMS protected hex-5-yn-1-ol (**2**) and 1-iodooct-2-yne (**1**), derived from 1-bromooct-2-yne, the diyne was prepared (**3**) applying Copper catalysis. The reduction to diene (**4**) was carried out under ambient pressure with H_2 and P2-nickel catalysis in 100% z-stereo-selectivity. After removing the TBDMS-protecting group, the free alcohol was oxidized with DMP under mild conditions in CH_2Cl_2. The reaction was observed by GC-MS until its completion, followed by filtration through a short Florisil-column. The filtrated reaction mixture was then evaporated to dryness and the residue was resolved and filtrated in pentane to give pure (5*Z*,8*Z*)-tetradeca-5,8-dienal (**5**) as a colorless liquid with strong smell. B) MS spectrum of (5*Z*,8*Z*)-tetradeca-5,8-dienal measured on a GC-Ion Trap-MS.

Fig S3. NMR characterization of the unsaturations in the *C. extensa* pheromone. A) Selective TOCSY (in red, upper spectrum) experiment irradiating the resonance at δ_H 5.35 with a Gaussian pulse of 100 Hz width and 30 ms mixing time showed neighboring resonances at δ_H 2.90, 2.26 and 2.19 appeared. Below, in black is the proton spectrum of the pheromone. B) Homodecoupling experiments saturating the resonances at δ_H 2.90 and 2.26 and extraction of the coupling constants (10.8 and 11.1 Hz) from the simplified double bond multiplet, showing that both unsaturations were *cis*-configured.

Fig.S4. 2D-spectrum of the long-term ^{1}H-^{1}H COSY NMR measurement.

Fig. S5. 2D-spectrum of the long-term ^{1}H-^{13}C HSQC measurement.

Fig. S6. A) Scheme showing the selected COSY correlations for and B) final assignment of the pheromone molecule, identifying it as (5*Z*,8*Z*)-tetradeca-5,8-dienal.

Fig. S7. Superimposed ^{1}H-NMR spectra of the synthetic (black) and isolated (red) *Corimelaena extensa* pheromone showed both compounds to be identical.

Fig S8. The pheromone can be detected in the headspace of feeding males but not of females feeding on *N. attenuata* cuttings. The pheromone was still present 90 min after removal from *N. attenuata* cuttings and transferal to a new flask to avoid contamination from previous release.

Fig. S9. Choice assay with excised flowers and immature seed capsules of the two tobacco hosts showed that both males and females prefer to feed on *N. attenuata* compared to *N. obtusifolia.* P-values represent differences in absolute number of individuals on each host in a paired Wilcoxon Test.

Fig S1.

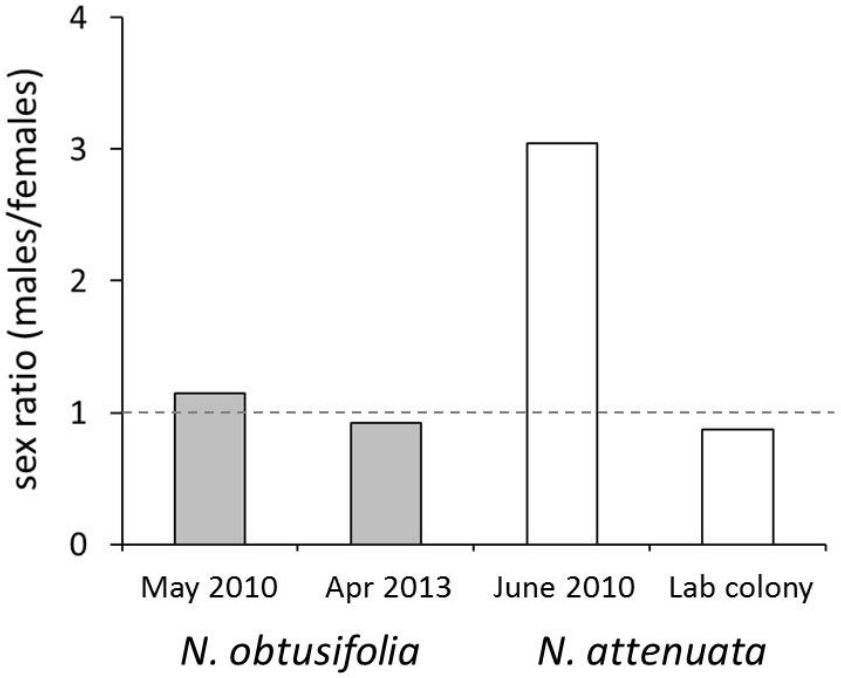

sex ratio (males/females)
4
3
2
1
0
May 2010
Apr 2013
June 2010
Lab colony
N. obtusifolia
N. attenuata

Fig S2.

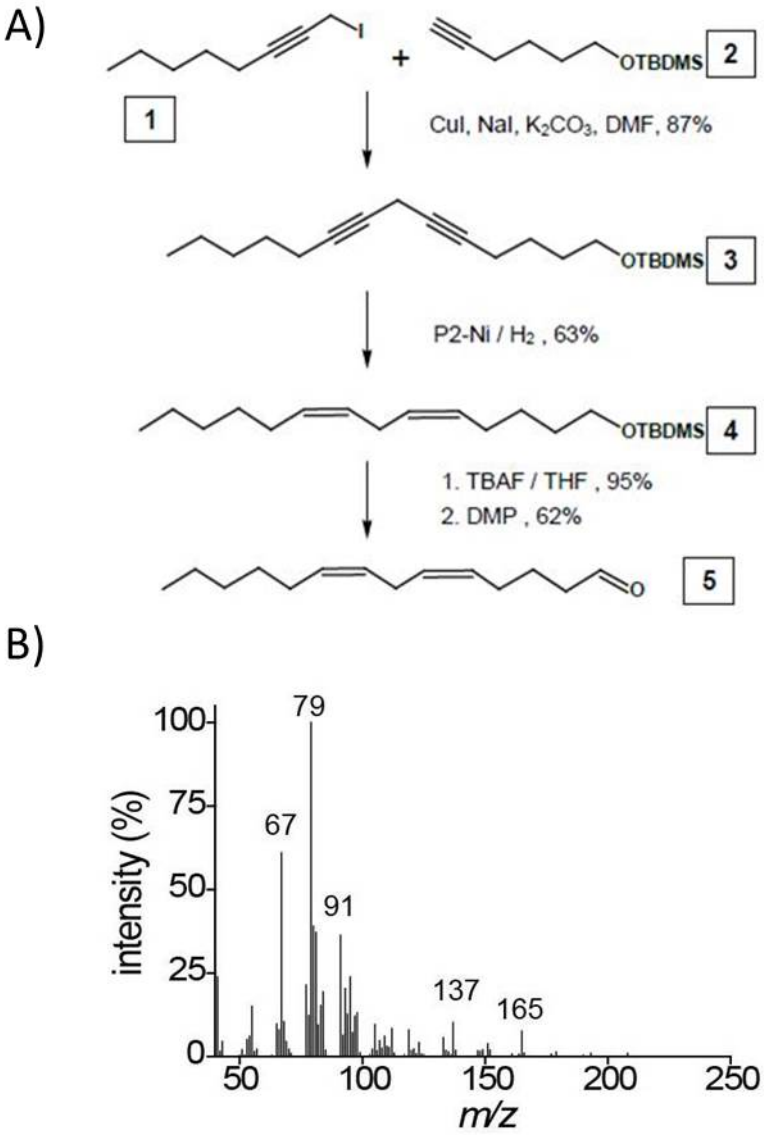

A)
1
+
OTBDMS
2
CuI, NaI, K2CO3, DMF, 87%
OTBDMS
3
P2-Ni / H2 , 63%
OTBDMS
4
1. TBAF / THF , 95%
2. DMP , 62%
O
5
B)
intensity (%)
100
75
50
25
0
79
67
91
137
165
50
100
150
200
250
m/z

Fig S3.

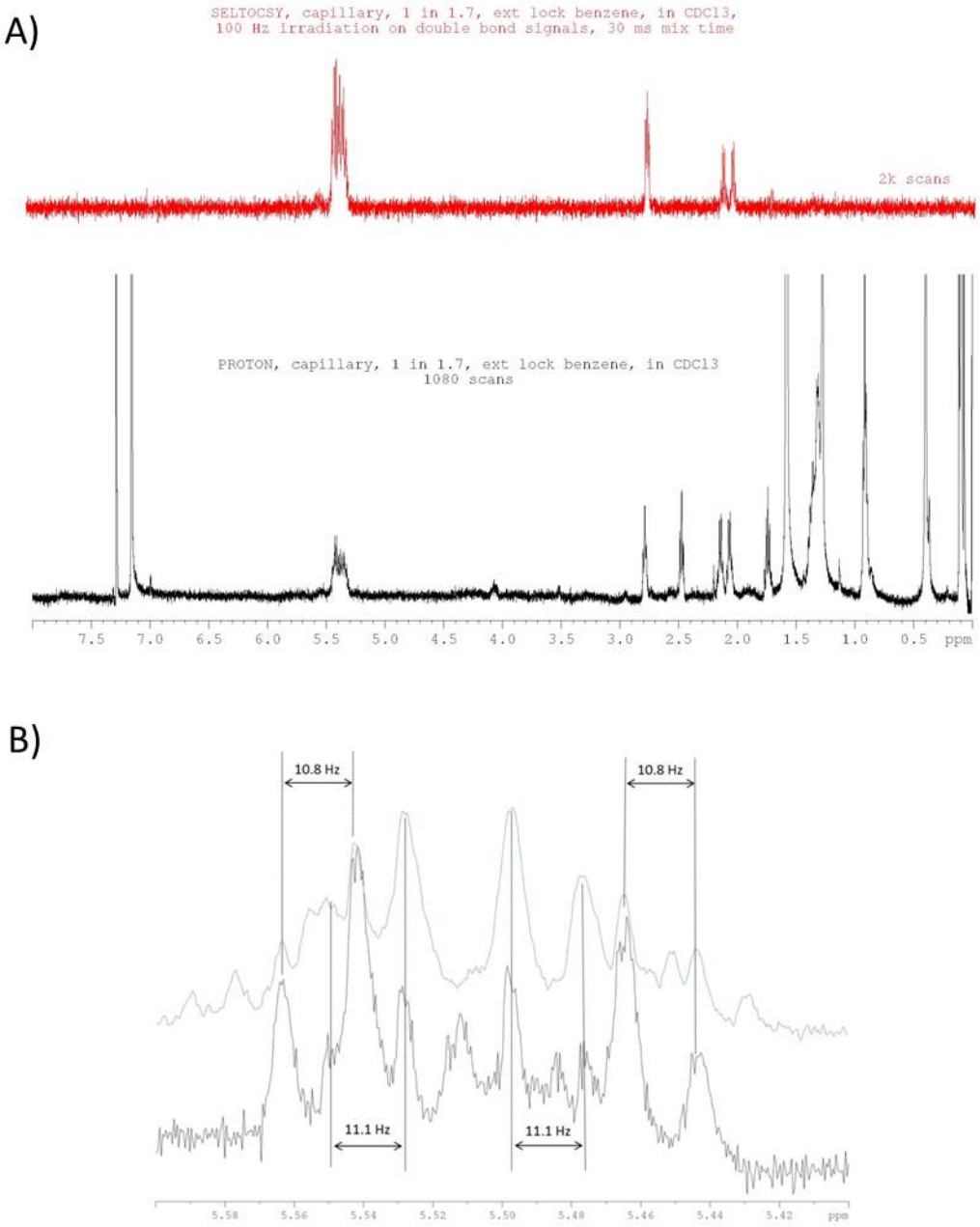

A)
SELTOCSY, capillary, 1 in 1.7, ext lock benzene, in CDCl3,
100 Hz irradiation on double bond signals, 30 ms mix time
2k scans
PROTON, capillary, 1 in 1.7, ext lock benzene, in CDCl3
1080 scans
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
B)
10.8 Hz
10.8 Hz
11.1 Hz
11.1 Hz
5.58 5.56 5.54 5.52 5.50 5.48 5.46 5.44 5.42 ppm

Fig. S4.

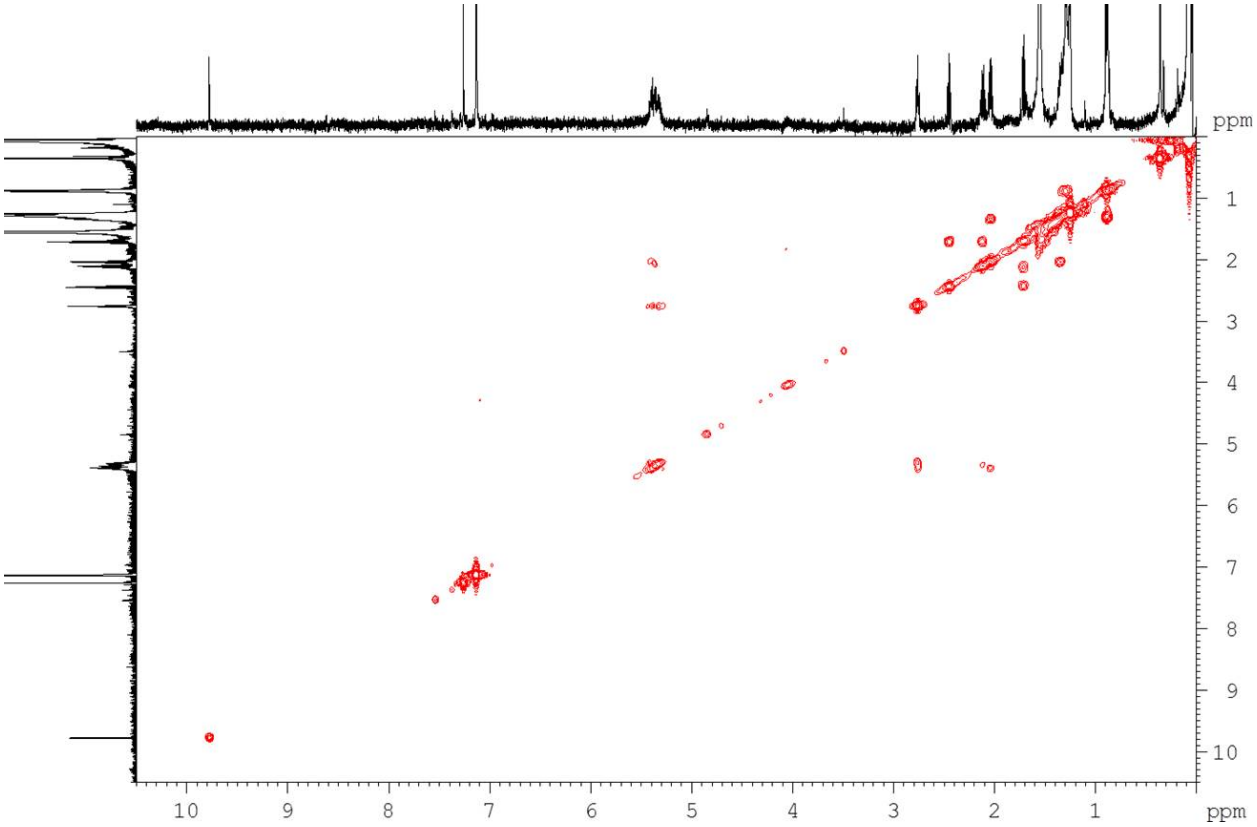


Fig S5.

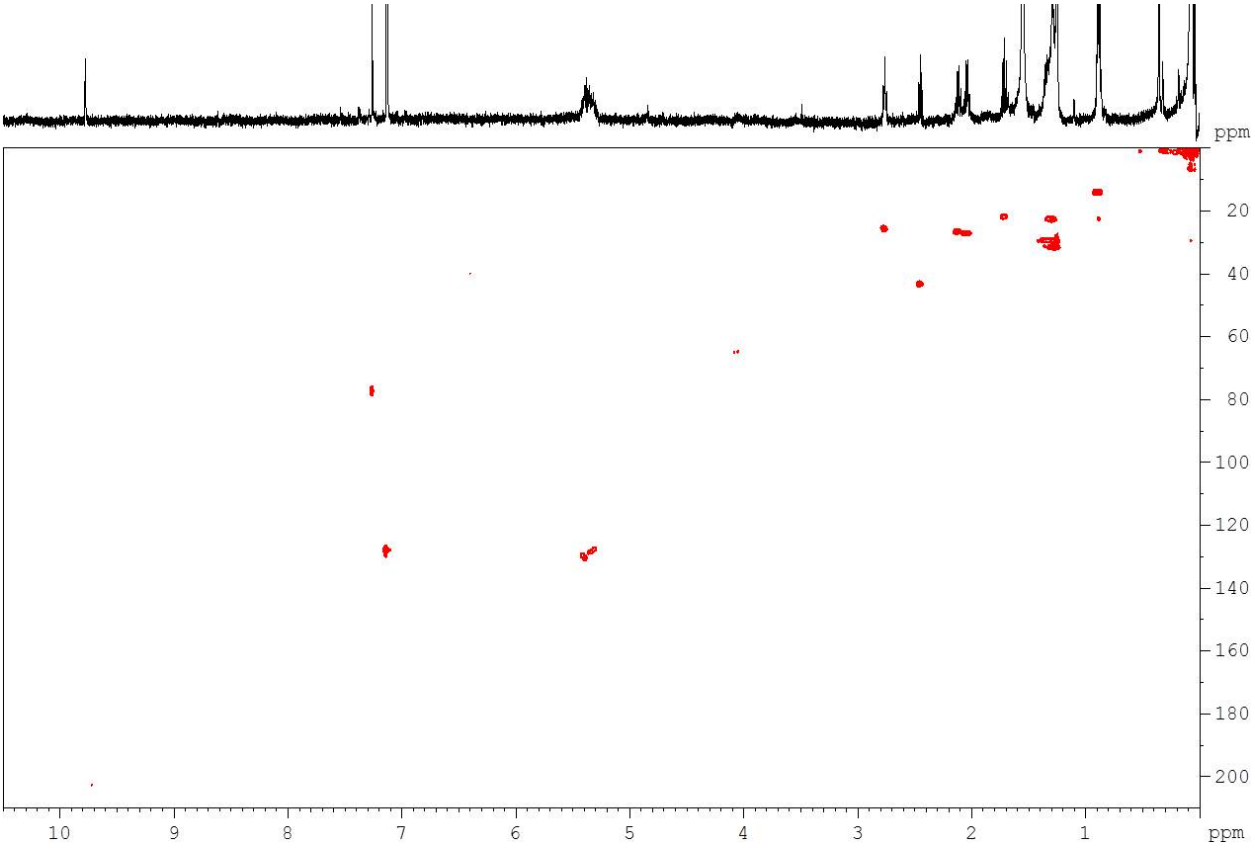
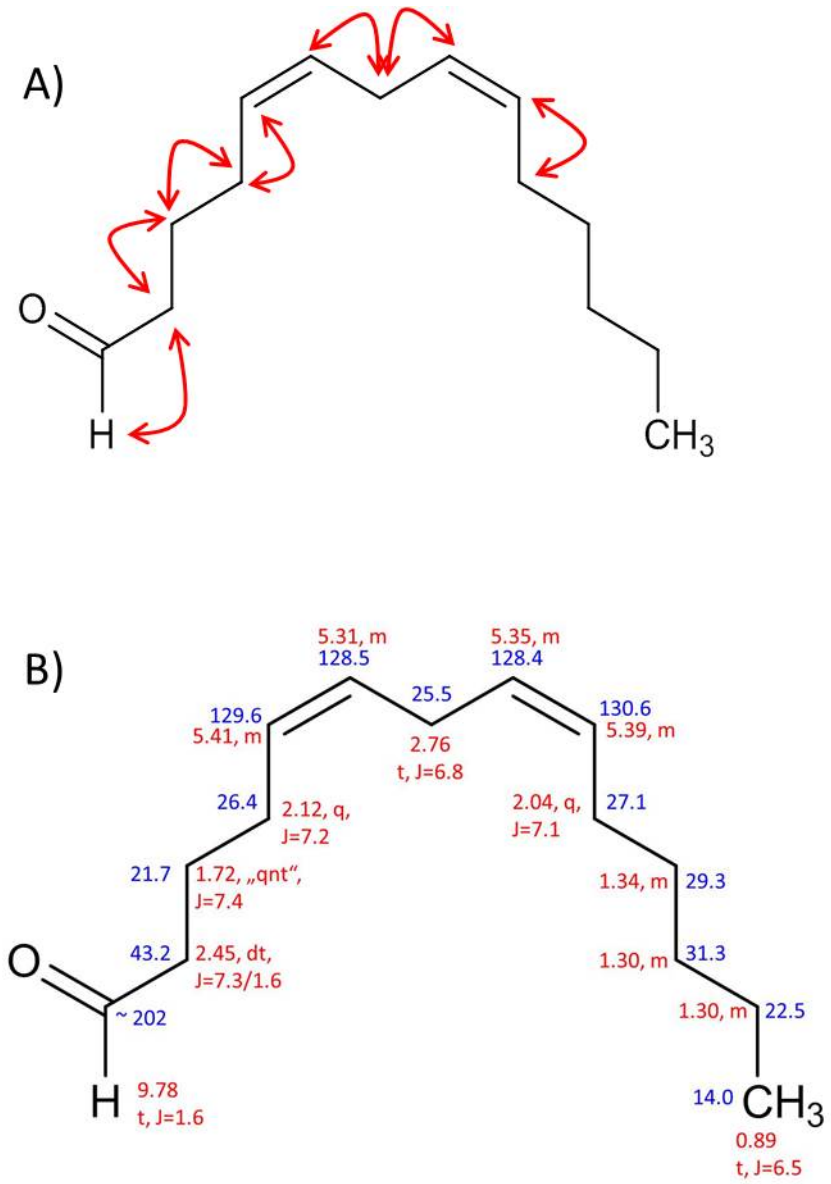


Fig. S6.


A)
O
H
CH3
B)
5.31, m
128.5
5.35, m
128.4
25.5
129.6
5.41, m
2.76
t, J=6.8
130.6
5.39, m
26.4
2.12, q,
J=7.2
2.04, q,
J=7.1
27.1
21.7
1.72, „qnt“,
J=7.4
1.34, m
29.3
O
43.2
2.45, dt,
J=7.3/1.6
1.30, m
31.3
~ 202
1.30, m
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CH3
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t, J=6.5

Fig. S7.

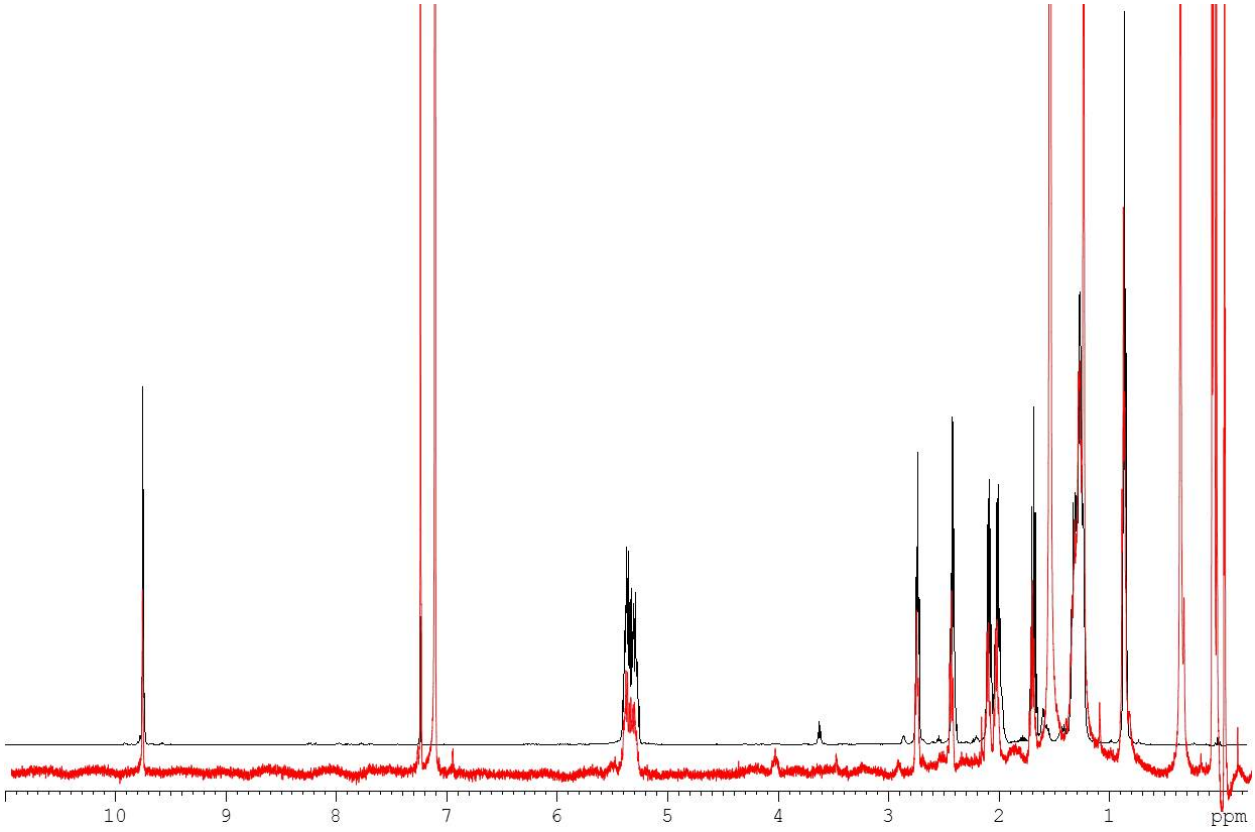


Fig. S8.

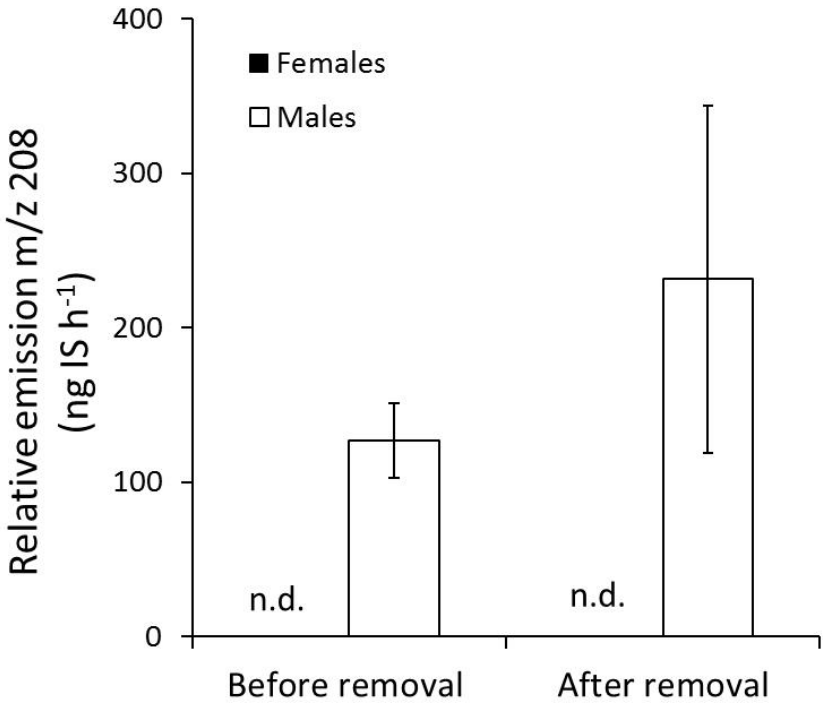
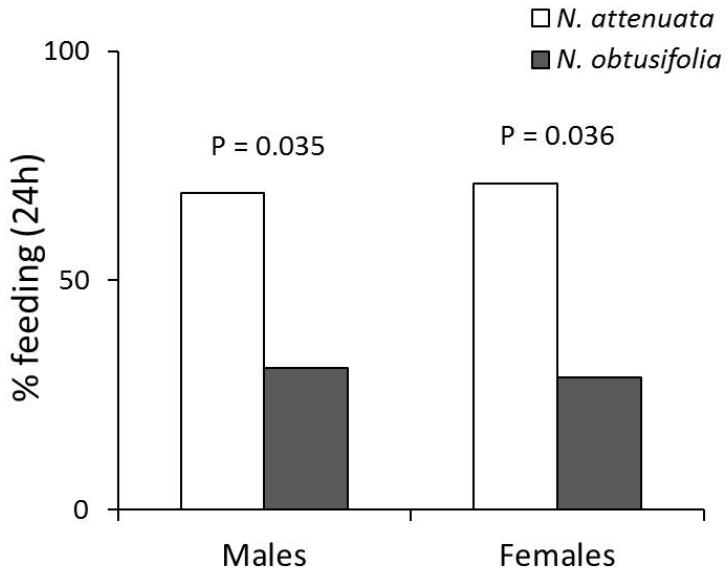

400
300
200
100
0
Females
Males
Relative emission m/z 208
(ng IS h-1)
n.d.
n.d.
Before removal
After removal

Fig. S9.


N. attenuata
N. obtusifolia
100
50
0
% feeding (24h)
P = 0.035
P = 0.036
Males
Females

4. Discussion

The aim of my dissertation was to explore the roles of phenotypic plasticity in the interaction of the post-fire annual *Nicotiana attenuata* with two insect herbivores of different feeding guilds, the lepidopteran herbivore *Manduca sexta* and the hemipteran seed predator *Corimelaena extensa*. Since the interactions of *M. sexta* and *N. attenuata* are well-studied, and N availability in the ephemeral post-fire habitat of *N. attenuata* is an important constraint on both its growth and defences, this system was an excellent model to study the effects of resource allocation on plasticity in plant induced defences using a new method for quantifying these trade-offs at the level of single compounds. This method is described in **manuscript I**, and examples of its application are given in **manuscripts II** and **III** and further discussed. In the final chapter (**manuscript IV**), I discuss the effects of resource availability in affecting both host plant life history and insect behavioural plasticity in the interaction of *N. attenuata* with the *C. extensa*.

4.1. A novel analytical method for dissecting resource allocation

Traditionally, the costs of defence induction have been quantified by measuring changes in photosynthesis rates, plant biomass, size and/or yield associated with an increase in defence metabolites (Bazzaz *et al.*, 1987; Karban and Baldwin, 1997; Baldwin and Hamilton, 2000; Zangerl *et al.*, 2002). But measurements of biomass don't distinguish between investments into growth-, defence-, and waste-related compounds, and it has been argued that growth-defence trade-offs should rather be measured as investments into single growth or defence-related compounds (Chapin III *et al.*, 1990). Additionally, in order to visualize these trade-offs it is necessary to measure them in the currency of a growth-limiting resource (Mole, 1994; Baldwin *et al.*, 1998), however the comparison of compounds with widely differing masses such as large proteins and small defensive metabolites is not possible using a single analytical method and, ideally, the methods for measuring each class of compounds should be comparable. In **manuscript I** of this thesis, I show a novel LC-MS based analytical method for quantifying proteins and measuring ^{15}N incorporation in a high-throughput manner and with accuracy comparable to existing methods for measuring small metabolites (e.g. Gaquerel *et al.*, 2010). This method allows for the quantitation of the leaf

proteome with unknown ^{15}N incorporation levels based on the use of a single unlabelled internal standard (bovine serum albumin, BSA), which makes it less expensive and generally applicable, since there is no need of synthesizing standard peptides for every protein of interest. Apart from being more reproducible and sensitive and less labour-intensive than traditional two-dimensional gel-based electrophoresis methods used for identifying and isolating proteins from complex samples, it allows for measurements of plant proteins cultivated in ecologically relevant conditions (soil-grown plants) as opposed to stable labelling experiments which require a set of unlabelled control plants and are usually performed in hydroponic or cell cultures (Gouw *et al.*, 2010; Kline and Sussman, 2010; Jehmlich *et al.*, 2010). This is especially important, since it has been shown that *N. attenuata* plants cultivated under hydroponic conditions have altered allocation to seed capsules, which are typically used as a measure of Darwinian fitness (Baldwin *et al.*, 1998).

4.2. Phenolamide biosynthesis mediates N-based growth-defence trade-offs at multiple levels

Although the costs of induced jasmonic acid (JA) responses have been shown in terms of reduced biomass or fitness (Baldwin, 1998; van Dam and Baldwin, 1998; Redman *et al.*, 2001; Zavala and Baldwin, 2004; Cipollini, 2007), few studies have analysed the within-leaf allocation to growth versus defence, mostly due to the lack of an adequate methodology for comparing N incorporation into proteins and small defence metabolites at the single compound scale. In **manuscript II**, I used the new LC-MS^E method described both above and in **manuscript I** to analyse N investments into single growth- and defence-related compounds after simulated herbivory by wounding and addition of the oral secretions (OS) of the specialist herbivore *M. sexta*. I additionally used a line silenced in the first step of JA biosynthesis (ir*LOX3*) and one silenced in the biosynthesis of phenolamides (ir*MYB8*) to disentangle the effects of JA induction with those of two of the three most abundant N-containing defence metabolites of *N. attenuata*: caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS).

The results show that OS elicitation causes N reallocation at multiple scales within the plant. In particular, I show evidence that OS elicitation decreases whole-shoot N investment and increases the concentration of N in roots of WT plants, but these changes are not observed in the shoots of ir*MYB8* and ir*LOX3* (roots were not analysed). The strongest N

reallocation effects were observed in young rosette leaves, which were wounded in the sink stage and showed a large decrease in total soluble proteins (TSP) and RuBisCO, and an increase in nicotine, CP and DCS in WT plants. Interestingly, these within-leaf patterns were maintained at an attenuated level in ir*LOX3* plants, but in ir*MYB8* plants the decrease proteins was decoupled from the herbivore-response. Since it has been shown that ir*LOX3* plants have reduced JA (Allmann *et al.*, 2010) and ir*MYB8* has a normal JA-burst (Kaur *et al.*, 2010), it is unlikely that JA is responsible for the observed N allocation patterns, and this suggests that MYB8 itself has an effect on remobilization of N from RuBisCO. Thus, the attenuated levels of N allocation response observed in ir*LOX3* are likely due to a reduced induction of defences, caused by the lower JA levels. The JA-regulated defence response includes induction of MYB8 and results in ir*LOX3* having an intermediate phenotype to ir*MYB8* and WT plants shown in **manuscript I**. The effect of MYB8 on protein turnover after OS elicitation may be direct, in a mechanism similar to the induction of N-assimilation genes caused by other R2R3-MYB transcription factors in algae, Scots pine and *Lotus japonicus* (Miyake *et al.*, 2003; Gomez-Maldonado *et al.*, 2004; Imamura *et al.*, 2009). Alternatively, MYB8 may indirectly affect the regulation of protein pools after herbivory through one of its many phenolamide products, some of which are present in very low abundance (Kaur *et al.*, 2010; Onkokesung *et al.*, 2012) and could potentially have signalling functions. Phenolamides have been previously shown to interact with RNA and DNA (Bassard *et al.*, 2010), suggesting a possible mechanism for the regulation of N allocation by *N. attenuata* MYB8 products.

Through use of a ^{15}N pulse-chase kinetic experiment, the work in **manuscript II** also demonstrated evidence that the N invested into phenolamide biosynthesis after OS elicitation did not come from RuBisCO turnover, despite the large decrease in RuBisCO observed in WT plants (approx. 90% decrease in elicited young rosette leaves), which theoretically releases sufficient N for the biosynthesis of these compounds. The rapid and high labelling of CP and DCS suggest that they are biosynthesized from recently assimilated N, possibly nitrate, since plants can store N assimilated as nitrate in their vacuoles (Millard, 1988). **Manuscript III** supports these conclusions, showing that a transgenic line of *N. attenuata* silenced for the expression of RuBisCO (as*RUB*) produces similar amounts of CP and higher amounts of DCS in the young elicited rosette leaf compared to WT plants. These as*RUB* lines also show increased nitrate pools in control (unwounded) leaves compared to WT, although these pools are lower than the total N invested into DCS after elicitation and detailed turnover

experiments are necessary to support the hypothesis that nitrate is the source of phenolamide biosynthesis. These results disagree with previous studies on RuBisCO-silenced lines of cultivated tobacco, *N. tabacum*, which found a decrease of nicotine and (non-polyamine conjugated) phenolic compounds in RuBisCO-silenced lines despite higher levels of foliar nitrate (Matt *et al.*, 2002; Stitt and Krapp, 1999). However, these studies were conducted in the absence of herbivory, and it is likely that in the lack of damage induction, these plants may preferentially divert their N allocation to maintaining growth and reproduction instead of defence compounds. **Manuscript III** also showed that as*RUB* lines have a similar N content and TSP levels and the same post-OS elicitation dynamics as WT, suggesting that they reinvest free N, which was not invested into RuBisCO synthesis, into other proteins. Based on these results and the evidence of increased root N after OS elicitation mentioned above, it is likely that the decrease in proteins serves as tolerance mechanism. Such a scenario would be similar to the observed resource sequestration of Carbon to roots after herbivory by *M. sexta* (Schwachtje *et al.*, 2006) which was also shown to be JA independent. Resource sequestration is thought to be important against specialist defoliating herbivores such as *M. sexta* (Orians *et al.*, 2011), although studies of C and N allocation in other systems have shown influence of JA responses in this process in other solanaceous plant species (reviewed in Orians *et al.*, 2011; Gómez *et al.*, 2010; Gómez *et al.*, 2012).

Previous studies on *N. attenuata* demonstrated that methyl jasmonate (MeJA) elicitation resulted in a high investment in nicotine (Baldwin *et al.*, 1994; Baldwin *et al.*, 1998; Lynds and Baldwin, 1998), but the N investment into nicotine biosynthesis after OS elicitation did not significantly differ in any of the three lines analysed in **manuscripts II** and **III** compared to WT, and the proportion of recently assimilated N measured as the relative ^{15}N investment (see **manuscript II** Fig. 4, and **manuscript III** Fig 1) is lower than that invested into phenolamides. This may be caused by differences in labelling of precursor pools of these compounds, since nicotine is constantly synthesized in the roots and phenolamides are synthesized in the shoots. But it is also likely to reflect differences in MeJA and *M. sexta* OS elicitation, as the former has been shown to cause a much stronger nicotine response in *N. attenuata*. Furthermore, *M. sexta* is nicotine-tolerant and has been shown to use this plant alkaloid for its own defence against predators (Kumar *et al.*, 2013). Thus it is likely that *N. attenuata* redirects N investment away from the expensive alkaloid nicotine and into CP and DCS, which have been shown to be effective against this herbivore (Kaur *et al.*, 2010). This also agrees with previous studies showing that elicitation by *M. sexta* OS elicits an ethylene

burst that decreases the cost of unnecessary investment into nicotine (Voelckel *et al.*, 2001; vonDahl *et al.*, 2007). Nicotine also acts as a permanent N sink, and N invested into this defence in *N. attenuata* and *N. sylvestris* was not remobilized to other functions, even under N limitation sufficient to affect plant growth (Baldwin and Ohnmeiss, 1994; Baldwin *et al.*, 1998). Therefore it is also possible that the rapidly synthesized and dynamic phenolamides may also be a cheaper defence in terms of remobilization of N.

Leaf development also affected within-leaf pool sizes of defences and proteins (**manuscript II**), for e.g., rosette leaves which were mature at the time of elicitation suffered lower decreases in RuBisCO and TSP than young leaves, but also invested in higher absolute pools of defence metabolites. Although this result seemingly contradicts the Optimal Defence hypothesis (McKey, 1974; Rhoades, 1979; Stamp, 2003), which would predict higher defence levels in the younger leaf, the higher RuBisCO levels in the older leaf would probably make it more valuable for rapid regrowth than the younger leaf which had still not reached maturity at the time of elicitation. Additionally, changes in the relative concentrations of defences to nutrients (in this case, proteins) from leaf to leaf within a plant and within a single leaf, can serve to increase the variability encountered by herbivores which may be increase foraging effort and be more detrimental than constantly high levels of toxins, according to the Moving Target hypothesis (Adler and Karban, 1994; Rodriguez-Saona and Thaler, 2005; Underwood *et al.*, 2005; Stork *et al.*, 2009). On the other hand, the large increase in RuBisCO levels of the first systemic stem leaf (S1) in ir*MYB8* plants, compared even to ir*LOX3* plants (**manuscript II** Fig. 4), suggests that costs of inducing phenolamides decreases RuBisCO levels in future leaves and may have an impact on competitive growth.

In summary, in this part of my dissertation I showed that plant responses to herbivory are complex and operate at multiple scales within an individual plant and that whole tissue levels of a resource such as N do not necessarily reflect or illuminate the dynamics of compounds within the tissue. Leaf age and development status at the time of attack affects the relative N allocation into single growth and defence-related compounds in a seemingly optimal way, but further studies on herbivore behaviour are needed to elucidate the effects of within plant variability in N allocation on herbivore fitness. Furthermore, the method applied in this part of my dissertation is general and can be used to analyse a broad range of protein samples in different organisms and it paves way for the study of resource allocation effects on plant ecological interactions in a higher resolution than was previously possible. Future work will seek to combine this methodology with more ecologically-realistic experiments to

be able to disentangle other important effects on plasticity in plant resource allocation such as intraspecific competition and effects of different herbivores; although future studies should also take in count herbivore counter-adaptations to this plasticity.

4.3. Using known tools to dissect an unknown interaction

Intraspecific variation in plant volatile organic compound (VOC) emission may also serve as a mechanism to reduce plant apparency to herbivores (Takabayashi *et al.*, 1994; Köllner *et al.*, 2008; Schuman *et al.*, 2009; Bruce and Pickett, 2011). However, consistently emitted VOCs are necessary for predators and parasitoids to learn to associate herbivore presence with a reliable cue and these are frequently induced by the JA signalling cascade (Dicke, 2000; Allison and Hare, 2009; Dicke and Baldwin, 2010). This reliability in damage or herbivore induced VOC signals can be exploited by herbivores, both for host location and for avoiding hosts with competing conspecifics for themselves or their offspring, or as a mechanism of avoiding natural enemies (Dicke, 2000; Bruce *et al.*, 2005; Allmann *et al.*, 2013; Martinez *et al.*, 2013). One behavioural counter-adaptation to plant plasticity in volatile profile by foraging herbivores is the use of constitutively emitted and less variable VOCs to find the host, and the olfactometer assays in **manuscript IV** (Fig. 4A) suggest that both sexes of *C. extensa* are capable of using constitutively emitted VOCs to find both *N. attenuata* and *N. obtusifolia* and to distinguish between them, although in this case I used inbred lines of both hosts for the assay and the effects of natural variation in constitutive host VOC emission should be further explored. The results from treating native *N. attenuata* with MeJA (Fig 2 of **Appendix**) suggest that JA-induced VOCs do not affect host choice by *C. extensa* within a *N. attenuata* population; however, the effects of JA-induced VOCs in long range attraction of this herbivore still need to be investigated. Another solution used by insects to decrease the variability in host plant VOC signals is the use of insect-derived cues, such as pheromones, as a reliable indicator of herbivore presence, which facilitates formation of feeding- and mating-aggregations. However, pheromone cues can also be exploited by natural enemies of the herbivore, and there are still few studies analysing the costs and benefits of pheromone emission for herbivores in a natural context (Vet and Dicke, 1992; Aldrich *et al.*, 1997; Millar, 2005; Wertheim *et al.*, 2005). In **manuscript IV** I show that *C. extensa* uses a newly identified pheromone – (5Z,8Z)-tetradeca-5,8-dienal – emitted by male adult pioneers to find the unpredictable host plant *N. attenuata* and form conspecific aggregations on this host. I

also show evidence that *C. extensa* does not show a male-pioneered colonization of the closely related alternate host plant *N. obtusifolia*, and these differences can be traced back to differences in emission of (5Z,8Z)-tetradeca-5,8-dienal and parallel differences in preference and performance on both hosts (**manuscript IV**).

While it is known that *N. attenuata* and *N. obtusifolia* have different defensive metabolites which may influence the increased performance of *C. extensa* on the former host (discussed in **manuscript IV**), it is possible that differences in reproductive effort and primary metabolite content, such as sugars and proteins, of flowers and fruit between both hosts is responsible for the observed fitness differences. This is supported by the fact that *N. attenuata* seeds are heavier than *N. obtusifolia* seeds (Anssour *et al.*, 2009, manuscript IV-Fig. 5), presumably providing more resource to a seed feeder, as has been shown for cotton-stainer bugs, *Dysdercus* spp., in which specialist species had higher reproductive rates than generalist species, due to their adaptation to feeding only on trees from the Malvales that produced large oil and N-rich seeds than the host plants of generalist *Dysdercus* spp. (Derr *et al.*, 1981). *N. attenuata* seeds are also known to contain little nicotine and no trypsin proteinase inhibitors (Baldwin and Karb, 1995; Van Dam *et al.*, 2001), which suggests that by feeding on seeds, *C. extensa* may be avoiding induced plant responses. Additionally, a field performance assay on transgenic lines of *N. attenuata* independently silenced for JA signalling and perception and also in its main floral scent (benzylacetone, BA), green leaf volatiles, nicotine and trypsin proteinase inhibitors (TPIs) showed no effect on adult *C. extensa* fecundity (**Appendix** – Fig 1A). Furthermore, a comparison of *C. extensa* performance on two natural accessions of *N. attenuata*, an inbred line of the Utah accession used in all other experiments in this thesis (wt (UT)) and an inbred line of an Arizona accession which lacks TPIs due to a point mutation in the Na*pi* gene (Glawe *et al.*, 2003; Steppuhn and Baldwin, 2007) and respective transgenic lines silenced for TPIs in the UT background (ir*PI* (UT)) and with constitutive expression of TPIs in the AZ background (s*PI* (AZ) - see **Appendix**), showed a significant increase of *C. extensa* performance on wt(AZ) compared to wt (UT), but this performance was not affected by TPI content (**Appendix** – Fig 1B). However, during this experiment wt (AZ) produced significantly more reproductive units (buds, flowers and immature seed capsules) than wt (UT) (**Appendix** – Fig 1C), suggesting that *C. extensa* performance on *N. attenuata* is more affected by available nutrients than defence metabolites. The AZ accession has been shown to have a higher reproductive effort (in numbers of reproductive units) than the UT accession (Steppuhn and

Baldwin, 2007; Steppuhn *et al.*, 2008), although the primary metabolite content of fruit and flowers has not been analysed and may also be responsible for the observed phenotype. But more work is necessary to analyse the effects of primary and secondary defensive metabolites on *C. extensa* performance and host choice, and also whether *C. extensa* induces changes in host plant chemicals.

Regarding the observed variation in pheromone release by *C. extensa* on both hosts, previous studies in various insect taxa point to a higher intraspecific plasticity in pheromone amounts than in blend composition (Roelofs *et al.*, 1987; Schlyter and Birgersson, 1989; Johansson and Jones, 2007); and also to a higher intraspecific variation in emission of aggregation than of sexual pheromones (Schlyter and Birgersson, 1989). Variation in blend composition is frequently associated with a host plant shift and formation of host-races, as in the case of the fall armyworm, *Spodoptera frugiperda* rice and maize races, which have different host plant preferences and a maternally inherited altered ratio of the same sex pheromone components, suggesting the possibility of incipient speciation between races (Groot *et al.*, 2008). Variation in the blend composition of aggregation pheromones is also thought to be related to speciation in bark beetles (Symonds and Elgar, 2004; Symonds and Elgar, 2008), but a case of phenotypic plasticity in a pheromone blend is reported for a population of the bark beetle *Ips pini* feeding on three different pine species which shows evidence of host plant-mediated plasticity and is discussed further below (Aukema *et al.*, 2010).

Many factors can influence intraspecific variation in total release of pheromones, including starvation, humidity, age, body size and individual genetic variation (Schlyter and Birgersson, 1989; Landolt and Phillips, 1997; Bashir *et al.*, 2003; Johansson and Jones, 2007). Although behavioural plasticity is thought to change pheromone output, e.g. reduced emission in the presence of signalling conspecifics or natural enemies (Schlyter and Birgersson, 1989; Bashir *et al.*, 2003; Johansson and Jones, 2007), relatively few studies show consistent effects of host plant identity on quantitative pheromone release. One example is the study of Aukema et al. (2010) which correlated both quantitative and qualitative changes in the aggregation pheromone of *Ips pini* feeding on three different pine hosts. The authors show the ratio of the *de novo* synthesized pheromone (Ipsdienol) to host-derived monoterpenes was higher when *I. pini* fed on *Pinus strobus* than *P. banksiana* and *P. resinosa*, and this change was correlated with greater attraction of conspecifics to *P. strobus* in the study area. Interestingly, the colonization success of *I. pini* on *P. strobus* was lower

than on the other two species, despite increased attraction, and is thought to be correlated to the difficulty of overcoming the greater resin defences of this host tree compared to the other two pine species, so in this case higher emission of the pheromone is not indicative of host quality. The grain-boring beetle *Rhyzopertha dominica* also shows predictable changes in aggregation pheromone release when feeding on artificial diets made with tissues from different host plants on which it normally feeds (Edde and Phillips, 2007). The main diet-mediated changes in pheromone emission observed are a quantitative increase in release of the blend, with the highest emission found on beetles fed on wheat-containing diet; and this is related to a greater attraction of *R. dominica* to wheat compared to other (mostly wild) host plants and may cause its pest status on this crop. This study, together with the higher performance of *C. extensa* on *N. attenuata*, suggest that the higher emission of (5Z,8Z)-tetradeca-1,5-dienal of *C. extensa* feeding on this host may serve as a reliable signal of host quality. Additionally, greater pheromone emission has been shown to be related to increased perception by conspecifics at longer distances (Edde and Phillips, 2007; Andersson *et al.*, 2013), which would increase efficiency of pheromone communication on widely scattered host plants.

To summarize, plasticity in pheromone release by *C. extensa* seems to be consistent with plant identity and quality and may be a behavioural mechanism to overcome plant VOC plasticity and facilitate host location of *N. attenuata* by making this host more apparent to conspecifics. Furthermore the high spatio-temporal variability and shorter phenology of the preferred host, *N. attenuata*, may act against formation of *C. extensa* host races on *N. attenuata* and *N. obtusifolia*, since this hemipteran is likely to rely on *N. obtusifolia* as an alternate host in the absence of *N. attenuata* populations and also possibly in spring and autumn, when the latter host is not flowering (Introduction - Fig. 3). Additionally, mating experiments with field collected *C. extensa* adults from both *N. obstusifolia* and *N. attenuata* showed no evidence of population isolation and produced viable offspring, and our field observations of the timing of colonization of both hosts suggest that *C. extensa* populations migrate from *N. obtusifolia* to *N. attenuata* in May/June and between patches of *N. attenuata* during the summer (Stanton, M.A. unpublished results). The work shown in **manuscript IV** sheds light on the previously unknown ecology of *C. extensa* and opens the way for future studies to analyse the precise effects of host plant type on the regulation of emission of the *C. extensa* pheromone. Further investigation is still needed to dissect if this effect is due to differences in biosynthetic precursors between *N. attenuata* and *N. obtusifolia*, or selective

pressure by natural enemies against high release of the pheromone on *N. obtusifolia.* This work is also one of the few to show a consistent host-related plasticity in release of an aggregation pheromone by an insect species and evidence of its adaptive value in facilitating the colonization of a host plant on which this species realizes a greater fitness, which is further increased by aggregation and communal feeding. It is also the first characterization of an aggregation pheromone and one of the first ecological studies of a species of the relatively unknown Thyreocoridae family ("negro" or "ebony" bugs) of the Hemiptera order.

4.4.Conclusions

Phenotypic plasticity is crucial for survival in variable environments and can greatly affect species interactions, but it is often studied only on one side of an interaction. The overall picture becomes more complex when plasticity in induced defences optimized to one herbivore does not affect other important herbivores, as shown for JA responses in *N. attenuata*, which are important in defence and tolerance mechanisms against the leaf feeding *M. sexta* but seem to be less effective against the seed feeding *C. extensa*. Plasticity in N allocation also seems to be important for tolerance and defence against *M. sexta* and most likely affects *C. extensa* interactions with both *N. attenuata* and *N. obtusifolia*, but future work is necessary to unravel this newly described interaction. In this dissertation I give an example of how a combination of analytical chemistry and molecular biology techniques can shed light on ecological questions, and that knowledge of a system's natural history is essential to understand the variables that affect both sides of these interactions.

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5. Appendix

Appendix: Effects of Jasmonate signalling and defensive metabolites of *N. attenuata* on *C. extensa* performance and host colonization

In this section I present additional unpublished data of field experiments seeking to dissect the impact of some of *Nicotiana attenuata* major volatile organic compounds, the floral scent, benzylacetone (BA) and green leaf volatiles(GLVs), jasmonic acid (JA) biosynthesis and perception, and two major direct defenses (nicotine and trypsin proteinase inhibitors) on the performance of native *Corimelaena extensa.* Additional data of observations of *C. extensa* colonization of native *N. attenuata* and also of native *N. attenuata* induced with methyl-jasmonate (MeJA) treatment which induces JA responses in this plant are shown. These results have been added as an appendix to my thesis since they shed more light on the newly described interaction between *C. extensa* and *N. attenuata* and are relevant for the general thesis discussion and future perspectives.

5.1.Field feeding assay with *N. attenuata* lines impaired in defence and signalling

In May 2009, we performed a no-choice feeding assay on field-grown *N. attenuata* defence and signalling mutants to assess the effect of these traits on *C. extensa* adult fitness, measured as fecundity after one of week of feeding. We used a 22x inbred wild type (WT) *N. attenuata* accession from Utah (wt (UT)) as control and transgenic lines of the same accession impaired in BA emission (ir-chal), GLV biosynthesis (ir-hpl, ir-lox2), single steps of JA signalling (ir-lox3, ir-aoc) and perception (ir-coi), and nicotine (ir-pmt) or TPI (ir-pi)

accumulation via inverted repeat (ir) RNAi constructs and a hemyzygous cross between irlox2 x ir-lox3 (hereafter, ir-lox2x3) impaired in both GLV emission and JA accumulation. Additionally, we used a 7x inbred WT accession (wt (AZ)) naturally deficient in TPIs due to a mutated version of the Na*pi* gene and a transformed line of the AZ accession with a constitutive expression of the wt (UT) *pi* gene (s-pi (AZ)). For a summary of gene abbreviations, phenotypes of the transgenic lines and publications characterizing each line, see Table 1.

Seeds were imported under US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) notification number 07–341-101n, and the field experiments were conducted under notification number 06–242-3r-a2. All transformed *N. attenuata* genotypes mentioned above were used for experiments in the experimental field plot at the Lytle Ranch Preserve near Santa Clara, Utah in 2009. Plants were germinated and cultivated as described in (Kallenbach *et al.*, 2012). During the no-choice feeding assay, 10 adult *C. extensa* bugs (5 males and 5 females) were confined to a single branch with developing flower buds of plants of the above lines (n=4-7), using a perforated plastic bag (240x350 mm, JG Verpackungen, Schönheide, Germany) fastened with a twist tie. *C. extensa* fecundity was measured as the number of eggs laid after one week of feeding and additionally the final number of closed buds ("buds"), buds with protruding corollas ("elongated buds"), open flowers and green seed capsules on the branch were recorded and compared between genotypes with a Student's t test. The cumulative number of eggs at the end of the experiment on each transgenic line was compared to its respective WT accession using a Wilcoxon test.

Results for the performance assay are shown in Fig. 1. No significant differences were found between the number of eggs laid on transgenic lines of the UT accession and wt (UT)

(P > 0.05 for all lines, Fig 1A). We found a significantly higher number of eggs laid by *C. extensa*

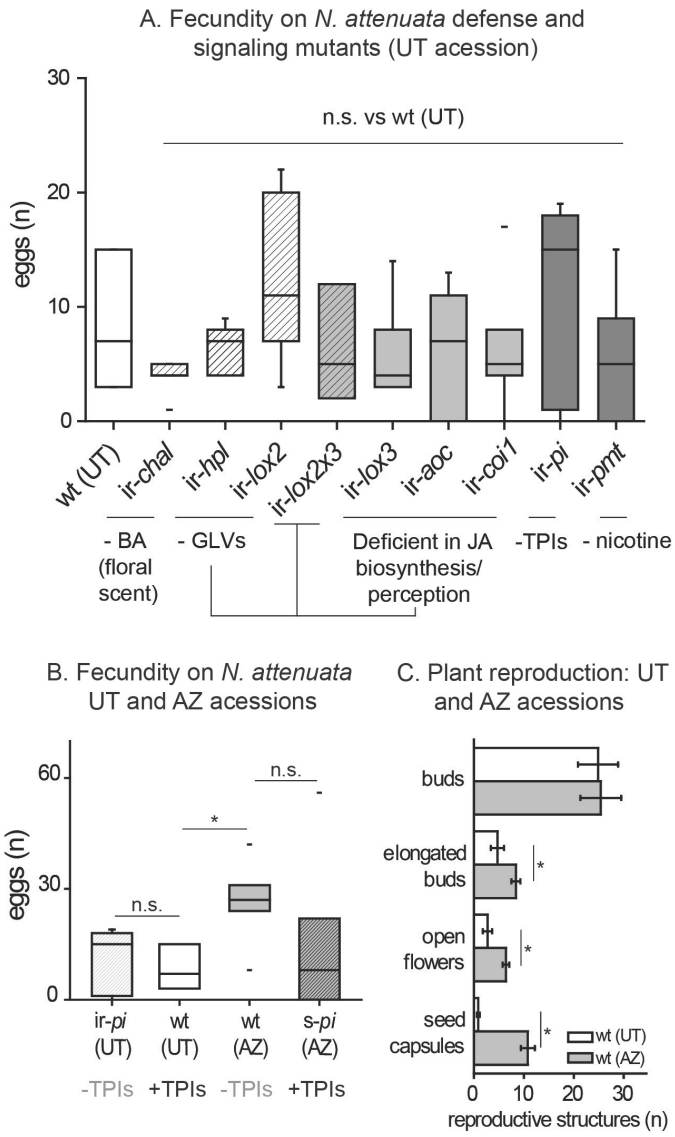

Figure 1. *C. extensa* adult fecundity is not affected by the major floral scent Benzylacetone (BA), Green Leaf Volatiles (GLVs), jasmonic acid (JA) biosynthesis and perception, nicotine and trypsin proteinase inhibitors (TPIs) of *N. attenuata* (**A**), but differs between two native *N. attenuata* accessions from Utah (wt (UT)) and Arizona (wt(AZ), which is naturally TPIs-deficient) (**B**). Fecundity was measured as the number of eggs laid by 10 adults (5 males + 5 females) after 7 days of feeding on field-grown plants. Data were analyzed with Wilcoxon tests (N = 4-7 plants), for comparisons between a single transgenic line and its respective WT accession. C) wt (AZ) produced significantly more elongated buds, flowers and seed capsules than wt (UT) (*= $p<0.05$ in Student's t tests). For gene abbreviations see Table 1.

feeding on wt (AZ) compared to wt (UT) (P = 0.018, Fig 1B). However, this difference in *C. extensa* performance does not seem to be related to differences in TPI content between these two accessions, since there were no significant differences between the number of eggs laid on a line silenced for TPI accumulation in the UT background (ir-pi) and wt (UT) (P = 0.68) neither between a line in the AZ background with recovered TPIs accumulation and activity and the wt (AZ) (P = 0.39) as shown in Fig 1B. Since the wt (AZ) showed a significantly higher amount of elongated buds (P = 0.048), open flowers (P = 0.017) and green seed capsules (P = 0.004) compared to wt (UT) on the branch used for the feeding assay (Fig. 1C) and bugs were restricted to a single branch, it is likely that the larger fecundity of *C. extensa* feeding on the AZ accession is due to larger availability of resources in the wt (AZ). Furthermore, previous studies comparing these two accessions have shown that wt (AZ) is a better competitor than wt (UT), with faster growth rates and higher reproductive output (Glawe *et al.*, 2003), but whether this also affects the nutrient quality (e.g. nitrogen or lipid content, or primary metabolite profile) of its seed capsules remains to be investigated.

5.2. Influence of JA-induced responses on the colonization of native *N. attenuata* by *C. extensa*

JA induced responses may affect host plant selection by phytophagous insects both through changes in the volatile emission of plants and through changes in host acceptance due to induced defensive metabolites (e.g. Kessler *et al.*, 2004; Halitschke *et al.*, 2008). Although most studied of JA-induced responses analyse changes in leaf defensive metabolites, it has been also shown that leaf damage can increase the nicotine content of *N. attenuata* new flowers and seed capsules

Table 1. Transgenic *N. attenuata* lines used for the *C. extensa* field feeding assay. Line name refers to the abbreviations used in the text, based on the altered gene name. Lines were silenced via inverted repeat (ir) RNAi or antisense (as) constructs and one line expressed the wt (UT) *pi* gene in sense orientation (s-pi). Publications in which these lines were characterized are also listed.

Line name	Gene name	Phenotype	Reference	Accession
ir-chal	chalcone synthase	impaired BA emission	(Kessler *et al.* 2008)	UT
as-hpl	hydroperoxide lyase	impaired GLV emission, enhanced JA induction	(Halitschke *et al.* 2004)	UT
ir-lox2	lypoxygenase 2	impaired GLV emission	(Allmann *et al.* 2010)	UT
ir-lox3	lypoxygenase 3	impaired JA biosynthesis/ accumulation	(Allmann *et al.* 2010)	UT
ir-aoc	allene oxide cyclase	impaired JA biosynthesis/ accumulation	(Kallenbach *et al.* 2012)	UT
ir-coi	coronatine insensitive 1	impaired in JA perception	(Paschold *et al.* 2007)	UT
ir-pmt	putrescine N-methyl transferase	impaired nicotine accumulation	(Steppuhn *et al.* 2004)	UT
ir-pi	trypsin proteinase inhibitors	impaired in TPI accumulation	(Steppuhn & Baldwin 2007)	UT
ir-lox2x3	hemizygous cross between ir-lox2 and ir-lox3	impaired in JA accumulation and GLV emission	Parent lines described above	UT
s-pi (AZ)	constitutive expression of wt(UT) *pi* gene	restored TPI accumulation	(Steppuhn & Baldwin 2007)	AZ

(Baldwin and Karb, 1995) and that methyl-jasmonate (MeJA) induction on leaves can significantly decrease damage to flowers and seed capsules in field grown plants up to one month after induction(McCall and Karban, 2006). Therefore we decided to investigate whether JA-induced responses may affect the colonization of *N. attenuata* by *C. extensa* adults. We used native plants in an isolated wash population of about 100 plants in May 2009 and induced 11 plants with 150µg of MeJA in 20 µL of lanolin paste on the abaxial side of one of the stem leaves as described in Kallenbach *et al.* (2012). Control plants were treated with 20 µL of pure lanolin paste (n = 11) and were size-matched to MeJA-treated plants. All plants were flowering at the time of treatment. Two weeks after treatment (3rd June), we recorded the number of feeding *C. extensa* adults on plants from both treatments and compared them with a Wilcoxon test. We found no difference in the number of adult *C. extensa* feeding on flowers and seed capsules of MeJA induced plants compared to controls (P = 0.767, Fig. 2), indicating the JA-induced responses don't affect the colonization of *N. attenuata* by these insects.

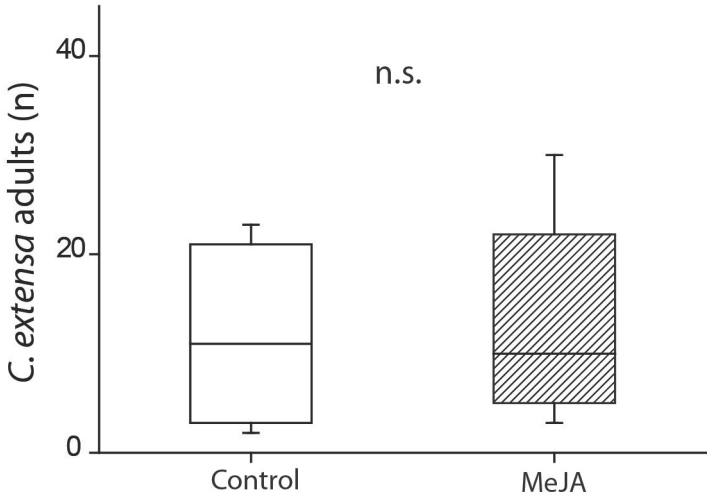

Figure 2. Colonization of native *N. attenuata* by *C. extensa* is not dependent on JA-induced responses. n.s. = P > 0.05 in a Wilcoxon test.

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6. Summary

Induced plant defences are an example of adaptive phenotypic plasticity in which plants only invest in defences when under herbivore attack, in order to spare limited resources for investment into growth and reproduction in the absence of herbivores. Additionally, phenotypic plasticity in plant metabolism can affect the abundance, distribution and performance of herbivores and their natural enemies, influencing community structure. The aim of my dissertation was to explore the roles of phenotypic plasticity in the interaction of the post-fire annual *Nicotiana attenuata* with two insect herbivores of different feeding guilds, the lepidopteran folivore *Manduca sexta* and the hemipteran seed predator *Corimelaena extensa*.

Due to its mass-germinating behaviour in response to cues from burned vegetation, *N. attenuata* grows under intense intra-specific competition for ephemeral nitrogen (N) in the burned soil and this growth-limiting nutrient is an ideal currency with which to explore trade-offs between growth related proteins and defensive N-containing metabolites of this species. In the first part of this dissertation I analyse N-based growth-defence trade-offs at multiple levels within *N. attenuata* after elicitation simulating *M. sexta* herbivory. For this I combined a novel LC-MS^E method for protein quantification and analysis on ^{15}N incorporation, together with a similar LC-based analysis of N-containing defence metabolites, and used two transgenic *N. attenuata* lines independently silenced for the jasmonic acid (JA) signalling cascade and for a transcription factor controlling the biosynthesis of N-containing phenolamides, Na*MYB8*. I present evidence that after elicitation *N. attenuata* may reallocate N from shoots to roots, but that the most drastic changes are observed at the leaf scale, with large decreases of N flux into proteins and an increase in defensive metabolites. I also show that these patterns do not depend on JA signalling, although JA is necessary for defence induction, and that the regulation of protein levels after elicitation is related to *MYB8* expression. Additionally, a ^{15}N flux experiment showed that N for the biosynthesis of the metabolically dynamic phenolamides does not come from degradation of ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO), the major foliar protein of *N. attenuata* and a putative N-storage protein, but likely from recently assimilated N. Further experiments on an *N. attenuata* transgenic line silenced for the expression of RuBisCO (as*RUB*) support these results, showing that as*RUB* plants accumulate similar levels of N-containing

metabolites (nicotine and caffeoyl-putrescine) and even higher levels of the phenolamide dicaffeoyl-spermidine as WT, despite having constitutively lower levels of RuBisCO.

The plasticity in the life-history strategy of *N. attenuata* which allows it to germinate from long-lived seed banks into an ephemeral N-rich habitat during a single growth season, makes it a resource which is unpredictable in both space and time. This makes *N. atenuata* a less apparent host plant for phytophagous insects which must disperse to find their host plants after overwintering elsewhere. In the second part of my dissertation I characterize for the first time the host-finding behaviour of the true bug *C. extensa* which feeds on the fire-chasing annual *N. attenuata* and the perennial *N. obtusifolia* in the Great Basin Desert in Utah. *C. extensa* consistently form larger aggregations on *N. attenuata* despite it being the less apparent of both hosts. In this work I identify the aldehyde (5Z,8Z)-tetradeca-5,8-dienal as the aggregation pheromone of *C. extensa* and through manipulative lab and field experiments I provide evidence that this insect exhibits a host plant-mediated plasticity in the release of this pheromone, relying more heavily on the pheromone for the colonization of *N. attenuata* than *N. obtusifolia*. I also show that *C. extensa* has higher realized fitness on *N. attenuata* compared to *N. obtusifolia*, and that the use of the aggregation pheromone on the annual host allows this insect to effectively manipulate host plant apparency to maximize its fitness. Lastly, I show evidence that JA responses and major defences of *N. attenuata* do not affect *C. extensa* fecundity or host choice, but differences between two *N. attenuata* accessions suggest that *C. extensa* fecundity positively correlates with plant reproductive effort and that by feeding on seeds this piercing-sucking insect may avoid JA-mediated plant defences entirely. Future studies will seek to disentangle the differences in host plant primary and secondary metabolism and higher trophic level effects which may affect insect fitness and influence the preference of these insects for *N. attenuata* over *N. obtusifolia*, despite increased costs of dispersal in order to colonize the former.

In summary, through use of a range of analytical chemistry techniques and isogenic lines modified for the expression of single plant traits, and a combination of lab and field work, I explored the plasticity on resource use by the post-fire annual *N. attenuata* and its effects on the interaction with two specialist insects with different feeding strategies, one of which has not been previously studied in an ecological context. I show that plasticity in plant responses to herbivory, including induction of defences and reallocation of N, are important in its interaction with the folivore *M. sexta*, but that the interaction with the seed predator *C. extensa* seems to be more strongly affected by resource allocation to reproduction rather than

induced defences. I also show that insect behavioural and physiological plasticity in the emission of an aggregation pheromone is important for the seed predator *C. extensa* to colonize the unpredictable host *N. attenuata* on which it realizes higher fitness. Future studies will seek to dissect the mechanisms through which host plants mediate this plasticity in herbivore pheromone emission.

7. Zusammenfassung

Ein Beispiel für Anpassungsfähigkeit und phänotypische Plastizität ist die induzierbare Verteidigung gegen Fraßfeinde in welche Pflanzen ihre Ressourcen nur im Falle eines Angriffs investieren, diese sonst aber für Wachstum und Reproduktion aufwenden. Die phänotypische Plastizität im Pflanzenstoffwechsel kann dabei Vorkommen, Verteilung und Effizienz der Pflanzenfresser sowie deren natürlicher Feinde beeinträchtigen und somit die Struktur der Biozönosen beeinflussen. Ziel meiner Dissertation war es, die Rolle der phänotypischen Plastizität in der Interaktion des zu den Feuerkeimern gehörenden einjährigen Tabaks *Nicotiana attenuata* und zwei seiner auf ihn spezialisierten Fraßfeinde zu untersuchen. Dabei ernähren sich die Larven des zu den Lepidopteren zählenden Schwärmers *Manduca sexta* hauptsächlich von Blättern während die Wanze *Corimelaena extensa* zu den Samenfressern zählt.

Durch Buschfeuer werden keimungsfördernde Substanzen freigesetzt, welche die massenhafte Keimung *N. attenuata* bedingen und folglich zu einem intensiven, intraspezifischen Wettbewerb um Ressourcen wie Stickstoff (N) führen. Aufgrund seines wachstumslimitierenden Effekts eignet sich N hervorragend um ‘trade-offs‘ (negative Abhängigkeiten) auf dem Niveau von wachstumsbezogenen Proteinen und N-haltigen Abwehrmetaboliten der Pflanzen zu untersuchen.

Im ersten Teil dieser Dissertation analysiere ich nach simuliertem Fraß durch *M. sexta* diese N-basierten ‘trade-offs‘ zwischen Wachstum und Verteidigung in *N. attenuata* mittels einer neuen LC-MS^{E}-Methode zur Proteinquantifizierung und Analyse von ^{15}N-Inkorporation. Zu diesem Zweck wurden weiterhin die ^{15}N-Inkorporation und die Quantitäten N-haltiger Abwehrmetaboliten analysiert. Dabei kamen transgene *N. attenuata* Pflanzen mit reduzierter Kapazität in der Jasmonsäure (JA) Signalkaskade und solche mit verminderter Expression des für die Biosynthese von N-haltigen Phenolamiden benötigten Transkriptionsfaktors Na*MYB8* zum Einsatz. Ich zeige, dass nach simuliertem *M. sexta* Fraß auf *N. attenuata* N von den Sprossen in die Wurzeln umverteilen kann, die drastischsten Änderungen jedoch innerhalb der Blätter stattfinden. Im Blatt wird der N-Flux in Proteine stark reduziert und jener in Abwehrmetaboliten erfährt eine Steigerung. Dabei hängt die Regulation der Proteinlevel nach der Fraß-Simulation mit der Expression von *MYB8* zusammen. Ich zeige weiterhin, dass diese Muster nicht von JA-Signalwegen abhängig sind,

wenngleich JA für die induzierte Abwehr benötigt wird. Mit einem ^{15}N-Flux-Experiment belege ich zudem, dass der N für die Phenolamid-Synthese nicht aus dem Abbau vom Ribulose-1,5-bisphosphat-carboxylase/-oxygenase (RuBisCO), dem häufigsten Blattprotein in *N. attenuata* und putativem N-Speicherprotein stammt, sondern viel wahrscheinlicher aus kurz zuvor assimilierten N. Meine Ergebnisse werden durch weitere Analysen transgener *N. attenuata* Pflanzen mit reduzierter Expression von RuBisCO (as*RUB*) gestützt, die zeigen, dass as*RUB*-Pflanzen zwar weniger RuBisCO als Wildtyp-pflanzen haben aber vergleichbare Mengen N-haltiger Metaboliten (Nikotin und Caffeoyl-putrescin) und sogar höhere Level für das Phenolamid Dicaffeoyl-Spermidin akkumulieren. Die Plastizität der Lebensstrategie die es *N. attenuata* ermöglicht aus langlebigen Samenbanken in ein vorrübergehend N-reiches Habitat zu keimen, macht diese Pflanze auch zu einer zeitlich wie räumlich unvorhersehbaren Ressource. *N. attenuata* scheint somit kein naheliegender Wirt für pflanzenfressende Insekten zu sein, da sich diese nach der Überwinterung zur Auffindung ihrer Wirtspflanze erst zerstreuen müssen.

Der zweite Teil meiner Dissertation konzentriert sich auf die Erstcharakterisierung des Wirtsfindungsverhaltens der Wanze *C. extensa,* welche sowohl auf der nach Buschbränden in der Great Basin Desert (Utah) keimenden einjährigen *N. attenuata* als auch der mehrjährigen *N. obtusifolia* vorkommen. Obwohl *N. attenuata* als der weniger wahrscheinliche Wirt erscheint, bilden sich durchweg größere Ansammlungen von *C. extensa* auf Pflanzen dieser Art. In dieser Arbeit identifiziere ich das Aldehyd (5Z,8Z)-tetradeca-5,8-dienal als das Aggregationspheromone von *C. extensa.* Mittels manipulativer Feld- und Laborexperimente zeige ich, dass dieses Insekt eine wirtspflanzenvermittelte Plastizität in der Freigabe des Pheromons aufweist und diese mehr für die Kolonisierung von *N. attenuata* als von *N. obtusifolia* benötigt. Ich zeige auch, dass *C. extensa* im Vergleich zu *N. obtusifolia* auf *N. attenuata* eine höhere Fitness realisiert und dass das Aggregationspheromon es dem Insekt erlaubt, die Auffindbarkeit der Wirtspflanze effizient für die Maximierung der eigenen Fitness zu manipulieren. Schließlich weise ich nach, dass die wichtigsten JA-vermittelten Abwehrmechanismen von *N. attenuata* keinen Einfluss auf die Wirtswahl oder Fertilität von *C. extensa* haben. Die Unterschiede zwischen zwei *N. attenuata* Akzessionen legen jedoch nahe, dass die Fertilität von *C. extensa* positiv mit dem Fortpflanzungsaufwand der Pflanzen korreliert und dass die Insekten durch ihre samenbasierte Ernährungsweise die JA-vermittelte Pflanzenabwehr vollständig umgehen können. Zukünftige Studien werden die Unterschiede im Primär-und Sekundärstoffwechsel der Wirtspflanze adressieren und mögliche Effekte auf

höheren Trophieebenen untersuchen, welche möglicherweise *C. extensa*'s Fitness beeinflussen. Die Ergebnisse könnten auch zu verstehen helfen, warum *N. attenuata* trotz erhöhter Kosten für die Auffindung und Besiedlung im Vergleich zu *N. obtusifolia* die bevorzugte Wirtspflanze für *C. extensa* darstellt.

Zusammenfassend erkundete ich in dem einjährigen Feuerkeimer *N. attenuata* mittels analytisch-chemischer Methoden, „isogenen" Pflanzenlinien und einer Kombination aus Labor- und Feldarbeit, die Plastizität in der Ressourcennutzung sowie deren Einfluss auf die Wechselwirkung mit zwei Insekten, die sich mit unterschiedlichen Fraß-Strategien auf diese Wirtspflanze spezialisiert haben. Ich zeige, dass die Plastizität der pflanzlichen Reaktionen als Antwort auf Insektenbefall, einschließlich der Induktion pflanzlicher Verteidigungen und N-Umverteilung, wichtig für die Wechselwirkung mit den folivoren Larven von *M. sexta* sind. Die Interaktionen mit samenfressenden *C. extensa* scheinen jedoch stärker von der Ressourcenbereitstellung für die Samenbildung als der induzierbaren Abwehr beeinflusst zu sein. Weiterhin zeige ich, dass die Plastizität in Verhalten und Physiology, bezüglich der Emission des Aggregationspheromons für *C. extensa* von großer Bedeutung ist, um die schwer vorhersehbar auftretende Wirtspflanze *N. attenuata* auf welcher die Insekten aber eine größerer Fitness realisieren können, zu kolonisieren.

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9. Erklärung

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena,

Mariana A. Stanton

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

Jena,

Mariana A. Stanton

10. Curriculum Vitae

Mariana Alves Stanton

Born 22nd May 1984, Rio de Janeiro, RJ, Brazil

Nationality: Brazil/United Kingdom

Research Experience

From 02/2009 – MPI-CE – Jena, Germany – Doctoral Thesis

Max Planck Institute for Chemical Ecology, Department of Molecular Ecology

Dissertation: "Coping with ephemeral resources and unpredictable hosts: nitrogen allocation in the post-fire annual *Nicotiana attenuata*, and a plant-specific pheromone used by its seed predator *Corimelaena extensa"*

Advisor: Prof. Ian T. Baldwin

2006-2008 – UNICAMP – Campinas, Brazil – MSc. Thesis

Universidade Estadual de Campinas (UNICAMP)

Dissertation: "Resource allocation in response to herbivory in the milkweed *Asclepias curassavica* (Apocynaceae: Asclepiadoideae)."

Advisor: Prof. José Roberto Trigo

2004-2005 – UNICAMP – Campinas, Brazil – Apprenticeship in Research (Equivalent to Bachelor Thesis)

Universidade Estadual de Campinas (UNICAMP)

Project: "Defense strategies against herbivory. Comparison between k- and r-strategists: *Ipomoea carnea fistulosa* (Convolvulaceae) and *Asclepias curassavica* (Apocynaceae: Asclepiadoideae)."

Advisor: Prof. José Roberto Trigo

2004 – UNICAMP – Campinas, Brazil –Research internship

Universidade Estadual de Campinas (UNICAMP)

Project: "Aristolochic acids as chemical defenses of *Battus polydamas* (Lepidoptera: Papillionidae)"

Advisor: Prof. José Roberto Trigo

Teaching Experience

2011-2012 – MPI-CE – Jena, Germany

Max Planck Institute for Chemical Ecology, Department of Molecular Ecology

International Max Planck Research School for the Exploration of Ecological Interactions with Molecular and Chemical Techniques (IMPRS)

Lecture on "LC-MS and couplings" during the course "Analytical techniques for biologists" in the IMPRS and the Molecular Ecology department (internal course).

2006– UNICAMP – Campinas, Brazil

Universidade Estadual de Campinas (UNICAMP)

Lecture on "Defense allocation in plants" in the Plant Physiology III undergraduate course at UNICAMP.

Education

From 02/2009 – IMPRS/FSU – Jena, Germany – Doctoral Thesis

International Max Planck Research School for the Exploration of Ecological Interactions with Molecular and Chemical Techniques (IMPRS), with concurrent enrollment at the Friedrich Schiller University in Jena

5-18/11/2007 – IFS/MISTRA – Rio Clarillo National Reserve, Chile – Field Course

The International Foundation for Science (IFS)/ The Foundation for Strategic Environmental Research (MISTRA) International Field Course in Chemical Ecology of Plants and Insects. Reserva Nacional Río Clarillo, Chile.

2006-2008 – UNICAMP – Campinas, Brazil – Master of Science degree in Ecology

Programa de Pós-Graduação em Ecologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP)

2002-2005– UNICAMP – Campinas, Brazil – Bachelor and Teaching degrees in Biology

Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP)

Stipends and Awards

2009 – Doctoral stipend from the recruitment process of the International Max Planck Research School for the Exploration of Ecological Interactions with Molecular and Chemical Techniques of the Max Planck Institute for Chemical Ecology

2007- First prize for Oral Presentations at the V Brazilian Chemical Ecology Meeting. Londrina, PR, Brazil

2007 – The International Foundation for Science (IFS)/ The Foundation for Strategic Environmental Research (MISTRA) grant for the International Field Course in Chemical Ecology of Plants and Insects. Reserva Nacional Río Clarillo, Chile.

2006 - Two-year Master thesis grant awarded by the Fundação de Amparo à Pesquisa do Estado de Sao Paulo - FAPESP (State of São Paulo scientific funding agency)

2004 – One-year Apprenticeship in research grant awarded by the Programa Nacional de Iniciação Científica do Conselho Nacional de Pesquisa e Desenvolvimento, PIBIC – CNPq (Brazilian national research funding agency)

Language Skills

English Native speaker

Portuguese Native speaker

Spanish Moderate reading, speaking and writing

German Literate, basic speaking and writing

Jena,

Mariana A. Stanton

11. List of Publications

Scientific Articles

- Stanton, M. A., Preβler, J., Paetz, C., Boland, W., Svatoš, A., and Baldwin, I.T. (in preparation) Plant-mediated plasticity in emission of an aggregation pheromone provides a reliable signal for a native tobacco seed feeder to locate an unreliable host plant in the field.
- Stanton, M.A., Ullmann-Zeunert, L., Wielsch, N., Bartram, S., Svatoš, A., Baldwin, I. T. and Groten, K. (2013) Silencing ribulose-1,5-bisphosphate carboxylase/oxygenase expression does not disrupt nitrogen allocation to defense after simulated herbivory in *Nicotiana attenuata. Plant Signaling and Behavior* **8**(12): e27570
- Ullmann-Zeunert, L., Stanton, M.A., Wielsch, N., Bartram, S., Hummert , C., Svatoš, A., Baldwin, I. T. and Groten, K. (2013) Quantification of growth-defense trade-offs in a common currency: nitrogen required for phenolamide defenses is not derived from ribulose-1,5-bisphosphate carboxylase/oxygenase turnover. *The Plant Journal* **75**(3):417-429
- Morais, A. B. B., Brown, K. S., Stanton, M. A., Massuda, K F, and Trigo, J. R. (2013) Are Aristolochic Acids Responsible for the Chemical Defence of Aposematic Larvae of Battus polydamas (L.) (Lepidoptera: Papilionidae)? *Neotropical Entomology* **42**(6): 558-564
- Ullmann-Zeunert, L., Muck, A., Wielsch, N., Hufsky, F., Stanton, M. A., Bartram S., Böcker, S., Groten, K. and Svatoš, A. (2012) Determination of ^{15}N‑Incorporation into Plant Proteins and their Absolute Quantitation: A New Tool to Study Nitrogen Flux Dynamics and Protein Pool Sizes Elicited by Plant−Herbivore Interactions

Presentations

- Stanton M.A. *, Baldwin I. T. 2011. Finding mates and food: chemical cues in the *Nicotiana attenuata* - Corimelaena extensa interaction. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany
- Stanton M.A. *, Trigo, J. R. 2007. Defense Allocation in *Asclepias curassavica* (Apocynaceae: Asclepiadoideae). V Brazilian Meeting of Chemical Ecology. Londrina, PR, Brazil

Posters

- Goldberg J.K., Joo Y., Kim S.G., Meldau S., Schäfer M., Schuman M., Stanton M.A., Baldwin I.T. Plant Volatiles: from regulation to consequences. ICE Symposium, MPI for Chemical Ecology, Jena, DE, Sep 2013
- Stanton M.A. *, Baldwin I.T. A tale of two tobaccos: direct and indirect traits mediating host choice by Corimelaena extensa seed feeders. 12th IMPRS Symposium, Max Planck Institute for Chemical Ecology, Jena, DE, Apr 2013
- Wielsch N.*, Ullmann-Zeunert L., Hufsky F., Muck A., Stanton M., Bartram S., Böcker S., Groten K., Baldwin I.T., Svatoš A. Plants under stress: A novel approach for the absolute quantification of partially N-labeled proteins and the simultaneous determination 15N-incorporation. 46. Jahrestagung der Deutschen Gesellschaft für Massenspektrometrie, Deutschen Gesellschaft für Massenspektrometrie, Humboldt-Universität Berlin, Berlin, DE, Mar 2013
- Gaquerel E.*, Brütting C., Schäfer M., Stanton M., Ullmann-Zeunert L., Gulati J., Erb M., Schöttner M., Baldwin I.T. New tools for the *Nicotiana attenuata* system: 'Real Time' genetic manipulation in nature, transcriptome-metabolome networks, fluxomics, and new imaging procedures. SAB Meeting 2012, MPI for Chemical Ecology, Jena, DE, Oct 2012
- Stanton M.A.*, Baldwin I.T. Finding mates and food: chemical cues in the *Nicotiana attenuata* - Corimelaena extensa interaction. 14th Symposium on Insect-Plant Interactions, University of Wageningen, Wageningen, NL, Aug 2011
- Stanton M.A.*, Baldwin I.T. Resistance or attraction: what defines the interaction between *Nicotiana attenuata* and Negro Bugs? 9th IMPRS Symposium, MPI for

Chemical Ecology, Dornburg, DE, Feb 2010

- Silva-Brandão K. L.*, Stanton M.A., Solferini V. N., Trigo J. R. The role of secondary chemical compounds of Aristolochia in the host shift of Troidini butterflies. ISCE 21st Annual Meeting, 2005, Washington, USA.
- Stanton M.A., Caselli C.B.* Evidence of optimal foraging in an ant community. XXV Annual Brazilian Ethology Meeting. 2007. São José do Rio Preto, SP, Brazil.

Jena,

Mariana A. Stanton

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